Strategies to Improve Conventional Chemotherapy for Patients with Osteosarcoma

---

Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde

(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

von

Bernhard Robl

aus

Österreich

Promotionskomitee

Prof. Dr. Ian Frew (Vorsitz)

Prof. Dr. Dr. Bruno Fuchs (Leitung der Dissertation)

Prof. Dr. Dario Neri

Prof. Dr. Lubor Borsig

Zürich, 2017

To my family

To my love

1 Table of Contents

2 Summary

Osteosarcoma is a rare disease, yet it is the most common primary malignancy of bone and thus, responsible for a considerable number of deaths. With the introduction of multidrug chemotherapy in the 1970s osteosarcoma patient survival rates increased from a low 20% to approximately 60-75% if localized disease is present. In contrast, survival rates in case of metastatic osteosarcoma remains at a low 20% despite the use of multiagent chemotherapy. Modern standard osteosarcoma therapy starts with the histopathological confirmation of the disease and continues with neoadjuvant chemotherapy and surgical removal of the tumor tissue. Subsequently, adjuvant chemotherapy is initiated. Despite this multimodal approach, the absence of significantly improved patient survival rates for approximately four decades asks for novel strategies to improve osteosarcoma therapy. The present work deals with different approaches of how to potentially improve treatment efficacy, functioning at multiple levels.

First, the limited amount of patient samples requires researchers to use the available primary tissue as efficiently as possible. However, most often biopsied, chemotherapy naïve tissue is studied. Despite the larger availability of chemotherapy treated tissue, resected tissue is hardly studied due to potential influences of therapy on tumor biology. In course of this thesis we studied known and putative tumor suppressors in osteosarcoma tissue prior to and after chemotherapy. We showed that the use of chemotherapy treated samples is feasible and biologically relevant by confirming the prognostic value of known tumor suppressors such as p53 or PTEN in osteosarcoma. In addition, we demonstrated via immunohistochemical analysis of post-chemotherapy osteosarcoma samples that patient survival and therapy outcome is influenced by the presence of cytoplasmic p16 in tumors. In summary, post-chemotherapy osteosarcoma tissue samples possess research and clinical value and may be employed in future studies. Especially with respect to mechanisms of resistance, a better understanding of the impact of modern chemotherapy on disease biology is desired.

Drugs such as cisplatin (CDDP), doxorubicin (DOX) or methotrexate were successfully used against osteosarcoma during the last four decades. However, these highly efficient drugs also cause severe side effects and thus, limit the maximum applied dose. One way to increase local drug concentration without increasing systemic drug concentration is the intraarterial (i.a.) administration of drugs. Despite pharmacokinetic proof of higher local drug concentrations after i.a. infusions, earlier clinical trials comparing i.a. administrations of CDDP and intravenous (i.v.) administrations yielded conflicting results. To this end, we evaluated i.a. versus i.v. administrations of equivalent amounts of CDDP in a controlled, experimental study without clinical confounding factors. Using a murine osteosarcoma model feasible for multiple i.a. infusions of CDDP, a tumor inhibiting dose of i.a. CDDP was identified. Upon comparison, i.a. CDDP was superior in inhibiting primary tumor growth compared to i.v. infusions of CDDP. Furthermore, i.a. CDDP reduced the total number of lung metastases, potentially owing to the stronger inhibitory effect on the primary tumors. In conclusion, i.a. CDDP infusions, not only yielded superior local tumor control, but also demonstrated benefit in reducing total numbers of pulmonary metastases.

The third aim of this work was to improve therapy by targeting metastatic disease, the primary cause of death in osteosarcoma. For this purpose, we evaluated an EDA-targeted, immunostimulatory TNF-α (F8-TNF), which was shown to be efficacious against experimental soft tissue sarcomas. Using a syngeneic murine osteosarcoma model, we tested the efficacy of F8-TNF against metastatic osteosarcoma and its dependency on the route of administration. Despite the absence of an effect on the primary tumor, a marked reduction of metastases was observed, likely due to increased EDA expression of pulmonary metastases compared with the primary tumor and an increase in immune cells in the lung parenchyma. The route of administration had no major impact on treatment efficacy. Next, we developed an amputation model of syngeneic osteosarcoma to evaluate the efficacy of F8-TNF, alone and together with DOX. Treatment with F8-TNF, alone or in combination with DOX did not significantly inhibit progressed experimental osteosarcomas nor increase

immune cells in the lung parenchyma. Moreover, enlarged spleens were observed after the treatment with F8-TNF, pointing to an increase in production of immunosuppressive leukocytes to counteract the effects of F8-TNF treatment.

Ultimately, we demonstrated multiple approaches with great translational potential for use in the clinics against osteosarcoma. Based on our results, we suggest studying chemotherapy-treated osteosarcoma samples, especially with respect to studying mechanisms of resistance against common chemotherapy. In a second approach, we demonstrated the superior efficacy of i.a. CDDP over i.v. CDDP in treating experimental osteosarcomas. We believe that i.a. infusions of CDDP have great potential in tumor control, especially in the case of easily accessed osteosarcomas of the limbs. Therefore, future preclinical and clinical studies should re-evaluate the potential i.a. chemotherapy. At last, we also assessed the potential of F8-TNF against metastatic disease. Our results demonstrated efficacy against early disseminated disease, however, anti-metastatic efficacy was abolished in large, established pulmonary metastases. In summary, future studies against osteosarcoma, might evaluate the detailed mechanisms of action of (novel) drugs, and combine them in the best way possible to ultimately overcome tumor-induced resistances and improve therapy.

3 Zusammenfassung

Osteosarkome sind sehr seltene Krankheiten, das heißt weniger als 1 Patient pro 2000 Personen. Nichtsdestotrotz, zählen Osteosarkome zu den häufigsten, primären Knochentumoren und sind schuld an einer beträchtlichen Anzahl an Todesfällen. In den 1970igern begann man mit der chemotherapeutischen Behandlung von Osteosarkompatienten und verbesserte deren Fünfjahresüberlebensraten von 20% auf ungefähr 60-75% im Falle eines lokalen Osteosarkoms. Trotz Chemotherapie blieben die Überlebensraten von Patienten mit metastasierenden Osteosarkomen bei niedrigen 20%. Jede Osteosarkomtherapie beginnt heutzutage mit einer Biopsie um das Osteosarkom pathologisch zu bestätigen. Danach folgen neodjuvante Chemotherapie, das chirurgische Entfernen des Primärtumors sowie der Metastasen und zusätzliche Zyklen adjuvanter Chemotherapie. Trotz dieser aggressiven, multimodalen Therapie haben sich die Überlebenschancen der Patienten seit ca. vier Jahrzehnten nicht signifikant verändert. Aufgrund dieser Tatsache beschäftigen wir uns im Zuge dieser Arbeit mit der präklinischen Evaluierung klinisch leicht umsetzbarer Möglichkeiten um bestehende Behandlungs- und Prognosemöglichkeiten zu verbessern.

Da behandeltes Gewebe meist in größeren Mengen vorhanden ist, wollten wir durch Immunhistologie zeigen, dass auch die Analyse von behandeltem Gewebe wertvoll ist. Dazu verglichen wir unbehandelte Osteosarkomproben mit chemotherapiebehandelten Tumorproben. Insbesondere untersuchten wir bekannte und potentielle „Tumor Suppressors“. Mit dieser Analyse konnten wir das Wissen über p53 und PTEN, das durch Untersuchungen von unbehandelten Gewebeproben bereits bekannt war, anhand von behandelten Gewebeproben bestätigen. Zusätzlich identifizierten wir zytoplasmatisches p16 als einen neuen, prognostischen Biomarker für Osteosarkome. Diese Ergebnisse zeigten, dass auch die Analyse von chemotherapeutisch behandelten Gewebeproben möglich ist und zum besseren Verständnis der Osteosarkombiologie beitragen kann. Insbesondere bei der

Untersuchung von Resistenzmechanismen gegen moderne Chemotherapie kann die Analyse von behandeltem Tumorgewebe hilfreich sein.

Chemotherapeutika wie Cisplatin (CDDP), Doxorubicin (DOX) oder Methotrexate wurden in unterschiedlichen Kombinationen mit Erfolg zur Behandlung von Osteosarkomen eingesetzt. Allerdings ist der Einsatz jener Medikamente, aufgrund der dosisabhängigen, schweren Nebeneffekte, stark limitiert. Intraarterielle (i.a.) Infusionen bieten eine Möglichkeit die lokalen Medikamentenkonzentrationen zu erhöhen ohne dabei die maximale, systemische Gesamtkonzentration zu überschreiten. Pharmakokinetische Studien zeigten höhere intratumorale Wirkstoffkonzentrationen im Vergleich zu intravenösen (i.v.) Infusionen, jedoch konnten klinische Studien keine verbesserte Tumorkontrolle in Abhängigkeit des Verabreichungsweges demonstrieren. Aufgrund dieser Tatsache verglichen wir die Auswirkungen von i.a. und i.v. CDDP-Infusionen in einem Tiermodell. Mithilfe des Tiermodells können Einflüsse, welche in einem klinischen Umfeld nicht vermieden werden können, ausgeschlossen werden. Nach der Identifikation einer wirksamen Konzentration von i.a. CDDP, verglichen wir die Wirkung gleichwertiger CDDP-Konzentrationen in Abhängigkeit ihrer Applikationswege. Das Tumorwachstum wurde am stärksten durch i.a. CDDP gehemmt, während i.v. CDDP keine signifikanten Auswirkungen hatte. Nicht nur das Wachstum des Primärtumors wurde nach i.a. CDDP gehemmt, auch die totale Anzahl an Lungenmetastasen war nach i.a. CDDP am niedrigsten. Diese Ergebnisse bilden eine Basis für die (Re-)Evaluierung von i.a. Infusionen von neuen sowie bekannten Medikamente in zukünftigen präklinischen und klinischen Studien.

Das dritte Ziel dieser Arbeit war es, einen neuen Therapieansatz gegen Lungenmetastasen, der häufigsten Todesursache von Osteosarkompatienten, zu finden. Hierfür untersuchten wir ein Biologikum namens F8-TNF, ein Antikörperfragment, welches gezielt an EDA-Fibronektin bindet und mit TNF-α gekoppelt ist. Frühere Studien konnten bereits die hohe Wirksamkeit von F8-TNF gegen subkutane Weichgewebstumore beweisen. Aufgrund der hohen Wirksamkeit gegen primäres und erneutes Tumorwachstum testeten wir die Wirkung von F8-

TNF in einem Tiermodell eines metastasierenden Osteosarkoms. Obwohl keine Wirkung von F8-TNF auf das Tumorwachstum festgestellt wurde, kam es zu einer Reduktion der Lungenmetastasen, unabhängig vom verwendeten Applikationsweg (i.a. versus i.v.). Sehr wahrscheinlich ist dies auf die erhöhte Expression von EDA in den Lungenmetastasen und eine Zunahme an Immunzellen im Lungenparenchym von behandelten Tieren zurückzuführen. Als nächstes wurde die Wirkung von F8-TNF auf natürlich entwickelte, weit fortgeschrittene Lungenmetastasen untersucht. Selbst in Kombination mit DOX konnten diesmal keine signifikante metastasenhemmende Wirkung erzielt werden. In Übereinstimmung mit der ersten Studie konnte diesmal auch keine erhöhte Anzahl an $CD4^+$ und $CD8^+$ Immunzellen im Lungenparenchym identifiziert werden. Allerdings wurde nach einer Behandlung mit F8-TNF eine Vergrösserung der Milz entdeckt. Diese Beobachtung lässt auf eine Reaktion der Metastasen schließen, welche eine Stimulierung der Milz zur Folge hat und möglicherweise zu einer Immunhemmung führt, die das Tumorwachstum begünstigt.

Abschließend kann man sagen, dass die Untersuchung von chemotherapeutisch behandelten Gewebeproben biologischen als auch klinischen Wert hat. Speziell zur Untersuchung von Resistenzmechanismen herkömmlicher Therapien ist dies ein vielversprechender Ansatz. Weiters konnten wir eine verbesserte Tumorkontrolle demonstrieren, indem wir CDDP i.a. anstatt i.v. verabreichten. Insbesondere für Osteosarkome, welche an leicht zugänglichen Körperteilen auftreten, bieten i.a. Verabreichungen zusätzliche Behandlungsmöglichkeiten. Zukünftige präklinische und klinische Studien sollten das volle Potential von i.a. Verabreichungen untersuchen. Zuletzt untersuchten wir noch die Wirkung F8-TNF auf Lungenmetastasen. Unser präklinisches Tiermodell zeigte, dass Immuntherapie prinzipiell gegen metastasierende Osteosarkome verwendet werden kann, allerdings gilt es die stark immunhemmende Wirkung von Lungenmetastasen zu überwinden. Diese und ähnliche Mechanismen sollten in Zukunft durch die Verwendung von präklinischen, realitätsnahen Tiermodellen studiert werden um

optimale Medikamentekombinationen zu ermöglichen und eine Verbesserung der Therapie zu erzielen.

4 Abbreviations

AJCC ..... American Joint Committee on Cancer

APC ..... antigen presenting cell

CDDP ..... cisplatin

cdk4/6 ..... cyclin-dependent kinase 4/6

COSS ..... Cooperative Osteosarcoma Study Group

CT ..... computed tomography

CTL ..... cytotoxic T lymphocyte

cytP16 ..... cytoplasmic p16

DC ..... dendritic cells

DOX ..... doxorubicin

EDA ..... extra-domain A

EMT ..... epidermal-to-mesenchymal transition

EURAMOS ..... European and American Osteosarcoma study group

F8-TNF ..... EDA-targeted TNF-α

FGFR1 ..... fibroblast growth factor receptor 1

FISH ..... fluorescence in situ hybridization

i.a. ..... intraarterial

i.v. ..... intravenous

IC_{50} ..... half maximal inhibitory concentration

IGF ..... insulin growth factor

IL ..... interleukin

ILP ..... isolated limb perfusion

L-MTP-PE..... liposomal muramyl tripeptide phosphatidylethanolamine

MDSC..... myeloid-derived suppressor cell

MMP ..... matrix metalloproteinase

MRI ..... magnetic resonance imaging

MSH2 ..... mutS homolog 2

NADPH..... nicotinamide adenine dinucleotide phosphate

NGS..... next generation sequencing

PCR..... polymerase chain reaction

PET ..... positron emission tomography

PTEN..... phosphatase and tensin homolog

TACE..... TNF-α converting enzyme

Tc ..... Techneticum

TGF-β ..... transforming growth factor β

TLR4..... toll like receptor 4

TMA..... tissue microarray

TNF-α ..... tumor necrosis factor α

5 Introduction

5.1 Epidemiology of osteosarcoma

Osteosarcoma is the most frequently occurring primary malignancy of bone. Every year, approximately 3 new cases are diagnosed per 1 000 000 persons of a general population (1, 2). With respect to osteosarcoma incidence as a first cancer, a first peak is observed during adolescence (9-25 years; incidence of about 8 cases per 1 000 000 per year) and a steady increase with age in the elderly (> 60 years; about 4-5 cases per 1 000 000 persons per year) (1). Despite being a rare cancer, osteosarcoma is the most common malignancy of bone and thus, responsible for a considerable number of deaths. Especially in pediatric patients, bone tumors rank among the five deadliest cancers with a mortality of approximately 5 per 1 000 000 (3). Current 5-year overall survival rates are at approximately 60% dependent on the study, in case of localized disease (4). Nevertheless, 5-year overall survival rates have not changed since the introduction of multidrug chemotherapy in the 1980s and remained constant for more than three decades. If metastatic disease is present, 5-year overall survival rates are still at a low 30%, similar to before the introduction of multidrug chemotherapy (4).

Multiple risk factors are associated with the development of osteosarcoma. Throughout different age groups, male sex is associated with a higher risk (56%) for osteosarcoma as well as possessing a taller stature (1, 5). Another risk factor for developing osteosarcoma is Paget's disease- a disease linked to a loss of stable bone structure due to remodeling (6). Approximately 1% of people with Paget's disease will eventually develop osteosarcoma (7). Besides growth-related bone remodeling, genetic factors- especially loss of tumor suppressor genes, and therapeutic radiation are also known to increase the risk for osteosarcoma (8).

5.1.1 Classification of osteosarcomas

A hallmark, common to all osteosarcomas, is the production of tumor-osteoid ("immature bone") by the malignant tumor stroma (9), nevertheless, osteosarcomas can be subdivided into several classes according to their histological appearance and their anatomical site of origin (Table 1). Conventional osteosarcomas, the most common form of osteosarcoma (about 75% of diagnosed osteosarcomas), arise from the intramedullary cavity of the bone and are divided into seven subgroups. Osteoblastic (65%), chondroblastic (10%) and fibroblastic (9%) osteosarcomas are generally the most frequently observed forms of conventional osteosarcomas and named according to the composition of the predominant intratumoral matrix (10).

Table 1. Subtypes of osteosarcoma. Adapted from reference (11)

Histological classification	Anatomical classification	
Conventional osteosarcoma	**Cortex-associated osteosarcoma**	**Other primary osteosarcomas**
Osteoblastic	Parosteal	Low-grade (central)
Chondroblastic	Dedifferentiated parosteal	Osteoblastoma-like
Fibroblastic	Periosteal	Multicentric
Epithelioid	High-grade surface	Osteosarcoma of the gnathic bones
Giant-cell rich	Intracortical	Post-irradiation
Small cell		Disease associated (e.g. Paget's disease, fibrous dysplasia)
Telangiectatic		

5.2 Clinical characteristics and diagnostics

Before clinical diagnosis, osteosarcomas are usually characterized through symptoms such as local pain and swelling, often even for months, until a physician is visited, usually after the occurrence of trauma or pathological fractures (12). In suspicion of an osteosarcoma, evaluation of the full patient history, physical examination and plain radiography is performed

(13). Radiography aids in determining the osseous alterations due to the progression of an osteosarcoma and either an osteoblastic or osteolytic or a mixed phenotype can be observed. Formation of interspersed calcified islands can also be observed in areas of soft tissue. Commonly observed in radiographs is the so called Codman triangle which is marked by a triangular ossification of the periosteum in the border region of tumor and healthy tissue.

Subsequent to the evaluation of the locally affected region, magnetic resonance imaging (MRI) can be performed to detect malignant changes in the adjacent soft tissue and to scan for potential “skip lesions” (14). In order to systemically detect areas of active bone turnover, such as bone metastases, bone scintigraphy (BS) with ^{99m}Tc-labelled diphosphonate is commonly performed (15). Compared to plain radiography, BS is able to detect bone metastases up to 18 months earlier, as biochemical changes occur prior to morphological changes (16). Recent developments in imaging technology support the use of positron emission tomography (PET) scans with e.g. ^{18}F-FDG as a tracer in order to detect sites of active bone turnover, such as those present in osteosarcomas or bone metastases, rather than planar BS (17, 18). Especially in combination with computed tomography (CT) scans, PET/CT shows superior accuracy and sensitivity compared to BS in detecting bone metastases (17). Furthermore, CT scans of the lung are nowadays standard in order to detect potential lung metastases if those are suspected (19). Ultimately, a histopathological analysis via tru-cut-biopsies (or rarely open biopsies) is always performed in order to verify the diagnosis of an osteosarcoma through classifying the genetic make-up, the histological subtype, the produced osteoid or matrix as well as the proliferation of tumor cells (13, 20). An overview of established and novel imaging modalities in osteosarcoma is given in Fig. 2 (17, 21-23).

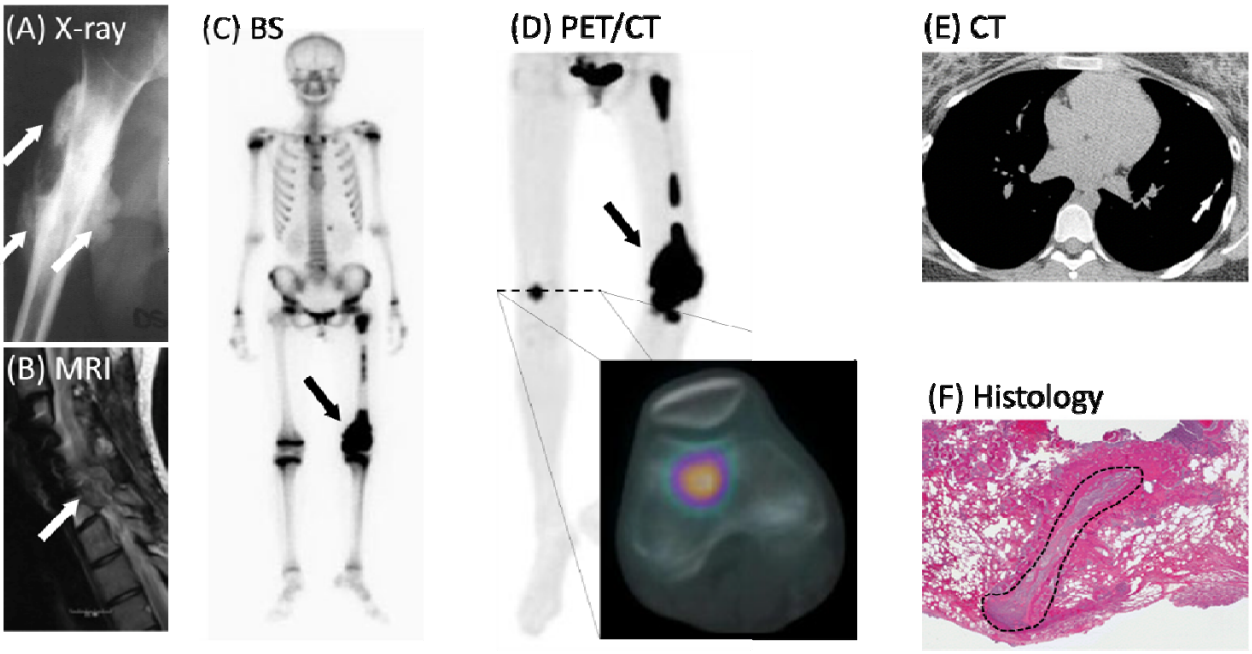


Figure 1. Overview of clinical imaging modalities used in osteosarcoma. (A) Plain radiograph of a femoral osteosarcoma of a 43-year-old female patient. White arrows depict osteoblastic lesions. (B) T2-weighted MR image showing an axial osteosarcoma metastasis compromising the spinal canal of a 20-year-old man. The arrow points at the tumor extending from the vertebra. (C) Whole body bone scintigraphy showing regions of activity in the left distal femur (arrow) of a 14-year-old female patient. (D) PET-CT scan of the same patient shown in (C). Arrow points at the primary osteosarcoma in the left femur. A bone metastasis is depicted in the epiphysis of the right femur, not detected by BS. The magnified image illustrates the overlay of the CT scan (white bones) and the PET-signal (colored area) shown as a horizontal section through the right knee. (E) CT scan of the lung of a 15-year-old girl, showing a pulmonary metastasis (white arrow). Bones and areas containing mineralized matrix are shown in white. (F) The same pulmonary metastasis as depicted in (E) is confirmed by histology (dotted region). The central osteoid matrix appears in blue and is surrounded by proliferating osteosarcoma cells.

After confirmation of the osteosarcoma, grading is performed. Most often the Enneking system which combines histological grades (low grade: stage I; high grade: stage II; and metastatic: grade III) and anatomical spread (within well-defined compartments: A; extending beyond surgical compartments: B) is used (24). In the US, the American Joint Committee on Cancer (AJCC) Staging System further distinguishes between tumor size, regional lymph node metastases and distant metastases(25).

5.2.1 Clinical markers

Upon diagnosis of osteosarcoma, a risk assessment based on known parameters ("markers") is performed to estimate patient survival. Standard chemotherapy consisting of highly cytotoxic drugs such as cisplatin (CDDP), doxorubicin (DOX) and methotrexate can induce severe side effects. Thus, it would be of great value to stratify patients before administering potentially ineffective but toxic chemotherapy. To this end, identifying the most important clinical markers is crucial.

The strongest single prognostic marker in osteosarcoma is the presence of metastases. If metastatic disease is present, five-year survival rates are at a low 16-29% (19, 26, 27). Not only presence of metastasis, also increasing numbers of detected metastases imply a worse prognosis (27, 28). Despite resection of macroscopic metastases, the disease has already spread microscopically and will, in most cases, progress later on (29). Apart from the presence of metastases, response to neoadjuvant chemotherapy is another strong prognostic factor. The histologic response is determined on the resected specimen and divided into good (>90% tumor necrosis) and poor responders (<90% tumor necrosis; a detailed classification of responders is found in Table 1). In general, good responders achieve up to two times better survival rates compared to patients with a poor histologic response (10, 19, 30). Furthermore, patient age, location of the tumor (axial versus limb), tumor size, anatomical location of the tumor (e.g. proximal versus distal) and the success of surgery also have prognostic value (31, 32). Besides poor histologic response, suboptimal chemotherapy (e.g. deviations from the standard protocol) is a chemotherapy-related factor for poor outcome (33). Surprisingly, other chemotherapy-related factors such as mild toxicities or infections are prognostic markers for improved survival of osteosarcoma patients (34-36). High dose chemotherapy is often associated with lymphopenia, leading to the attenuation of the immune system. However, the fast recovery of lymphocyte numbers is another, more recently discovered prognostic marker for good survival (37).

5.3 Biology of osteosarcoma

Osteosarcoma research suggests a close link between osteosarcoma development and processes involving bone growth and bone remodeling such as those taking place during puberty or bone-associated diseases. Consequently, most osteosarcomas (75%) arise in the metaphysis of the fastest growing long bones (humerus and tibia) of adolescents and young adults (8). As represented by the various manifestations of osteosarcoma (sub classifications are shown in Table 1), the actual cell of origin in osteosarcoma is still under debate (38). It was first believed that an omnipotent transformed mesenchymal stem cell (MSC) is causative for osteosarcomas, however, increasing experimental evidence supports the hypothesis that the cell of origin is derived from a pool of MSCs that underwent at least some degree of osteogenic-commitment (39).

However, until now no definitive proof for a specific unique cell of origin for osteosarcomagenesis has been identified. Eventually both models are valid to a certain degree, explaining the high diversity of osteosarcoma subtypes. Adding to the degree of complexity, another recent study also demonstrated a role of the tumor microenvironment in defining the ultimate outcome (i.e. osteoid-depositing versus osteoid-negative sarcomas/ cancers) of proliferating cells along the mesenchymal-osteogenic lineage after losing important tumor suppressors (40). In the end, different cells of origin under the influence of microenvironmental cues might ultimately induce osteosarcoma formation (38), yet at a different incidence (e.g. MSCs form osteosarcomas at lower frequencies compared to osteogenic progenitors) or with different genotypes (e.g. MSCs can form osteosarcomas after loss of a higher variety of tumor suppressors than osteogenic progenitors). This gap in understanding the origin of osteosarcoma renders osteosarcoma research more difficult (for instance in next generation sequencing (NGS) of tumor samples, where the lack of a proper control tissue hampers comparative analyses(41)).

In contrast to the uncertainty about the true origin of osteosarcoma, it is becoming accepted that osteosarcoma progression is supported by the action of osteoclasts causing a “vicious

cycle" of pathological bone remodeling (see Fig.1; adapted from (42), a concept which was already deposited in the context of other cancers e.g. prostate or breast cancer (43)). Subsequent to stimulation of mono-nucleated pre-osteoclasts by osteoblasts, activated T lymphocytes or osteosarcoma cells via RANK/ RANKL interactions, multinucleated (i.e. mature) osteoclasts form and start to resorb the bone matrix. This resorption process degrades the inorganic matrix and releases growth factors, among which insulin growth factors (IGF) and transforming growth factor-β (TGF-β) are most abundantly present (44, 45). In turn, the available growth factors and cytokines lead to a microenvironment that causes proliferation of osteosarcomas, which in turn propagate destruction of the host bone, allowing the tumor to grow and spread beyond its cortical boundaries (46).

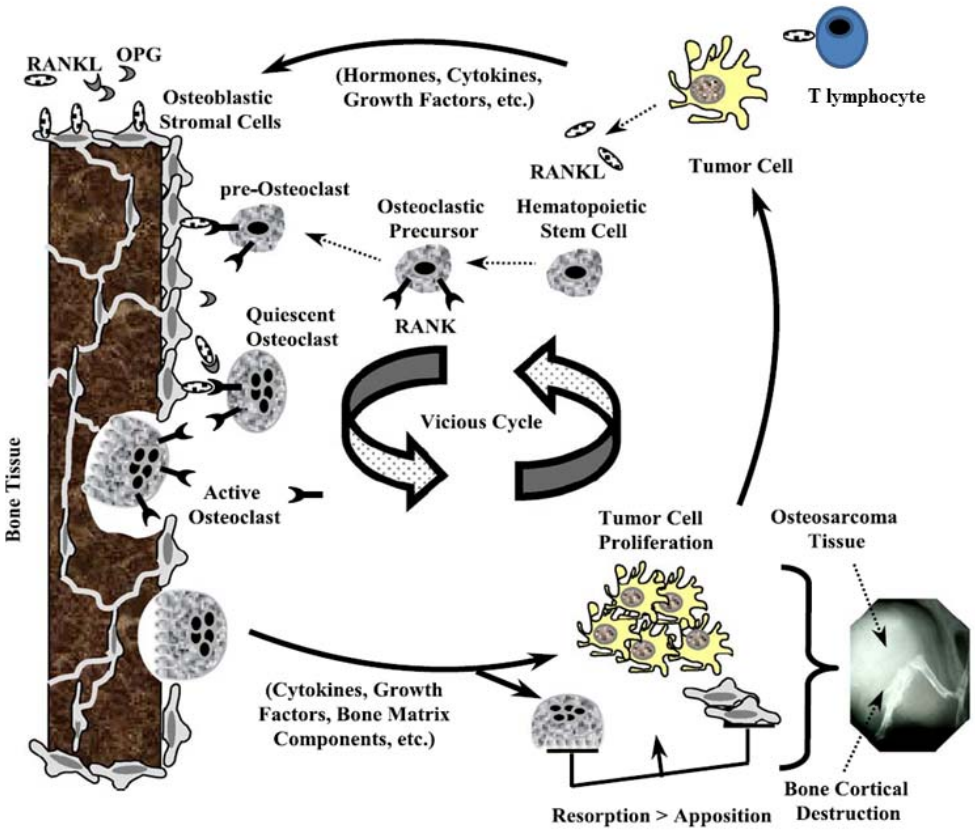


Figure 2. Illustration of the "vicious cycle", adapted from (42).

5.3.1 Genomic heterogeneity

Although the clinical course of osteosarcomas is remarkably consistent, the pathological appearance as well as the underlying genetic make-up is highly diverse. Despite multiple studies analyzing chromosomal abnormalities (47), copy number variations (48), exomes (49)or even whole genomes (50, 51), no consistent pattern besides an early or late loss of TP53 was detected (52). In contrast to Ewing's sarcoma, another class of bone cancers, no recurrent chromosomal translocation (such as EWS/Fli1 in the case of Ewing's Sarcoma) has been associated with initiating osteosarcoma development yet (53). Rather a pronounced genomic heterogeneity between osteosarcoma samples and, compared to other cancers, a high aneuploidy level is observed in studies of osteosarcomas (41). Using non-sequencing methods, copy number alterations of osteosarcoma samples were studied and increased aneuploidy with a seemingly non-random distribution of specific chromosomal gains and losses was detected. These generally high levels of genomic instability were shown to be associated with an expression signature of genes involved in cell cycle and replication (54).

Another phenomenon contributing to high genomic heterogeneity in osteosarcoma is termed chromothripsis. Chromothripsis is the process by which massive chromosomal rearrangements occur during a single cellular catastrophic event which statistically cannot be the consequence of the sequential accumulation of genomic alterations. Interestingly, chromothripsis was found to be especially frequent among osteosarcomas (33% of studied samples) and other bone cancers (55). These extensive chromosomal changes might further contribute to the genomic heterogeneity of this disease entity (41). A third frequently observed chromosomal event called kataegis- localized hypermutated regions, was found in over 50% of osteosarcoma samples (50). However, regions of kataegis were not associated with the most commonly mutated genes. Structural chromosomal variations rather than single nucleotide variants were associated with major dysregulated genes in osteosarcoma. Until now, the cause of these chromosome-changing events is not fully understood and mostly explained by statistical means, not yet by experimental proves. In conclusion,

osteosarcoma genomes are strongly heterogeneous due to genetic instability caused by multiple chromosomal lesions.

5.3.2 Molecular markers

In recent years, due to the trend of personalized medicine, molecular markers in cancer diagnosis achieved more and more interest in comparison with clinical markers. The current view, that every cancer is different, asks for a per-patient treatment defined by the unique genome/ molecular expression pattern of the individual tumors. Until recently, techniques such as PCR, comparative genomic hybridization, FISH, karyotyping or immunohistochemical techniques were mostly used to study these molecular markers in primary tumor tissue until NGS techniques became available and allowed for massively parallel analysis of genomic, transcriptomic or epigenomic data. On a genome level, already non-NGS studies revealed a correlation between genomic instability of osteosarcoma samples and metastasis-free survival of patients (54, 56) or the histological subtype of the tumor (57).

When looking at individual genes, conventional osteosarcomas are marked by a loss of heterozygosity (LOH) of *RB1* or mutations of *RB1* and either type of alteration was found in more than 60% of tumors (58). Furthermore, deactivation or loss of *TP53* is central in the development of osteosarcoma and thus, alterations are observed in approximately 50% of osteosarcoma samples (59). Similarly, loss of *CDKN2A* due to small deletions or copy number losses of *PTEN*, other major tumor suppressors, are detected in approximately one third of osteosarcomas (60, 61).

Besides loss of functions of these major tumor suppressors, simultaneous amplifications of several oncogenes or tumor promoting genes can be found in osteosarcoma samples. Among these alterations are amplifications of commonly known tumor drivers such as CDK4 (a cell cycle promoting kinase)(56) or mdm2 (an inhibitor of p53)(62). Another common tumor driver is VEGFA, an angiogenic growth factor required for solid cancers to induce angiogenesis, which is also associated with the progression of osteosarcoma (63).

Other known tumor drivers such as c-myc or c-fos are found to be strongly expressed in osteosarcomas (64, 65). Of these, expression of the osteoclastogenic transcription factor c-fos via induction of FGFR1 is considered to play an important role in the lung metastatic propensity of osteosarcomas (66). Especially IGF signaling plays an important role in osteosarcoma biology (67), by stimulating the formation of bone metastases (68), as well as increasing the formation of bone-resorbing osteoclast-like cells (69). In contrast to these common tumor drivers, overexpression of RUNX2 seems to be almost exclusively associated with the development of bone cancers or bone metastasis, apart from induction of lymphomas (70). This observation seems to confirm the critical role of the transcription factor RUNX2 in osteoblast differentiation and cell cycle regulation in osteosarcoma development.

Apart from the above described tumor drivers, metastasis associated genes have clinical value and are closely associated with patient survival. Among the most prominent metastasis associated genes in osteosarcoma is ezrin, a cytoskeletal linker protein (71). Upon phosphorylation, ezrin converts to an active conformation and moves to the cellular membrane where it helps stabilizing F-actin (72). Likewise, CXCR4, a chemokine receptor, was proposed to have important pro-metastatic functions in osteosarcoma (73, 74).

Nowadays, serological markers which can be measured using liquid biopsies (e.g. blood samples) are intensively studied due to their minimally-invasive quality. In osteosarcoma diagnosis, two molecular markers have often been shown to be significantly linked to poor survival, namely, high serum levels of alkaline phosphates (10) and increased levels of lactate dehydrogenase (32). In contrast to these two (phenotypical) protein markers, microRNAs (miRs) have showed great potential as liquid biopsies due to their circulatory availability and their regulatory influence on tumor progression at many stages. A recent analysis of patient survival-related miRs in osteosarcoma detected a subset of miRs that regulate prominent pathways involved in osteosarcomagenesis such as IGF, PTEN/ Akt/ mTOR, MAPK and PDGFR/ RAF/ MEK/ ERK (75). More recent studies involving NGS-techniques, showed that genes involved in osteosarcoma progression are regulated by

expression or suppression of certain miRs and thus, miRs are also considered as potential therapeutic targets besides their diagnostic value (76).

Finally, NGS studies contributed to the understanding of disease development and specific osteosarcoma treatment. First, NGS studies employing animal models and/or patient samples confirmed the contribution of many growth factor signaling pathways and their downstream kinases (e.g. PI3K-AKT-mTOR and ERK-MAPK (51, 77)) to osteosarcoma progression as well as the importance of mutations in *TP53* and *RB1* for osteosarcoma progression (50). Second, NGS studies discovered novel important driver genes in osteosarcoma progression. For instance, axon-guidance related genes such as *SEMA4D* and *SEMA6D* were identified as playing a tumor-promoting role in human osteosarcoma (77). Furthermore, *ATRX* (involved in telomere maintenance) and *DLG2* (implicated in polarity during cell division and cancer cell invasion) were found to be frequently mutated in human osteosarcoma samples (50).

In conclusion, earlier studies investigating proteomic and genomic markers of osteosarcoma progression were confirmed by more recent NGS-studies, laying the foundation for the major characteristics of osteosarcoma. Future studies will refine the knowledge about osteosarcoma by deciphering entire genomes and molecular expression patterns associated with specific tumor cells/ stages/ subtypes of osteosarcoma and if possible, individually for each patient.

5.3.3 Metastasis

As tumors grow and evolve, all malignant tumors will develop metastasis at early or late stages. Basically, metastasis describes a process where cells, after being shed from the primary tumor, leave the tumor bed and spread throughout the body, most often via the vascular systems to reach distant organs (depicted in Fig. 3 (78)).

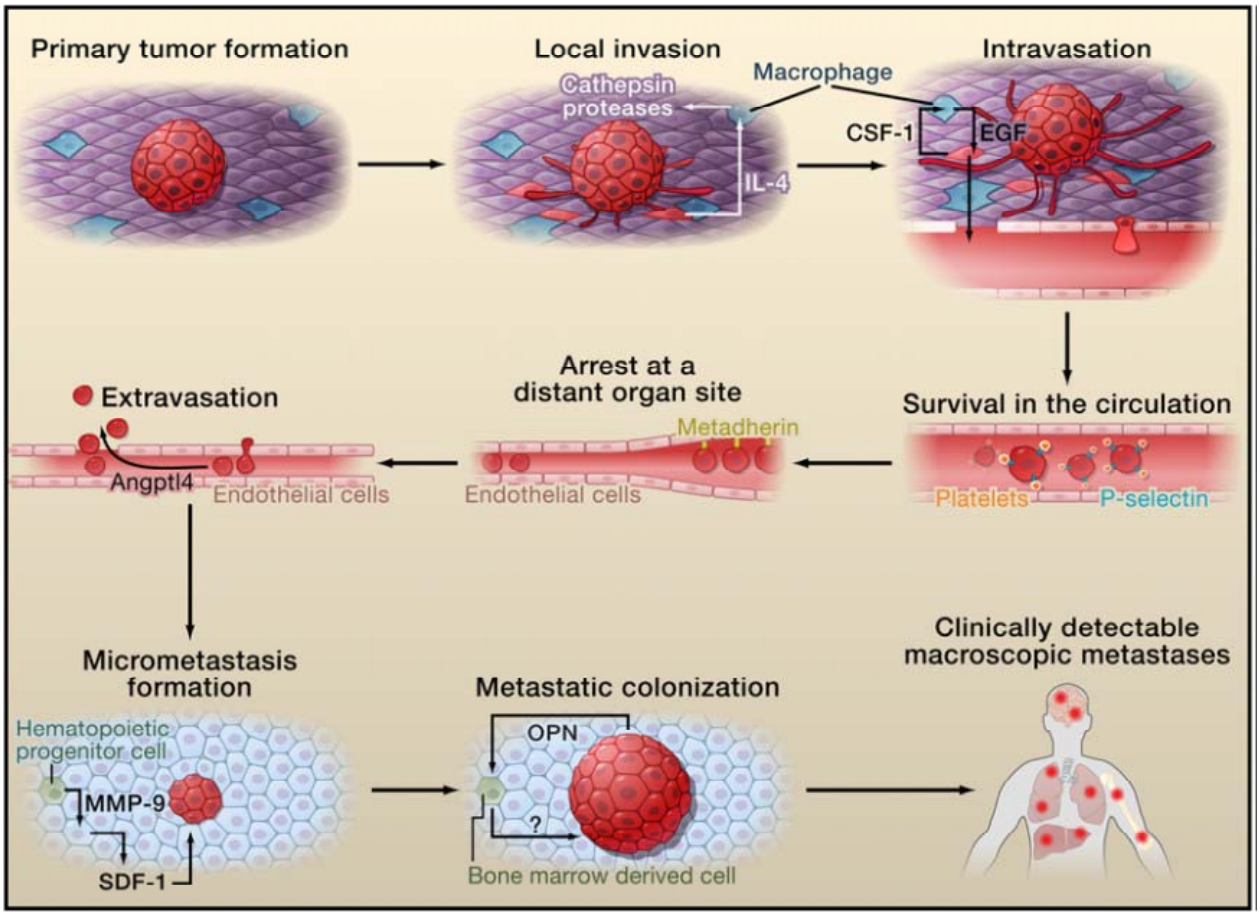


Figure 3. Steps of the metastatic cascade. After achieving unlimited proliferative capacity, cells of the primary tumor (1) leave the tumor bed and (2) intravasate into the circulatory system. After (3) surviving in the circulation cells need to be (4) arrested/ stop at a distant site. Subsequently halted tumor cells (5) extravasate through the endothelium and (6) initiate survival programs adapted to a new microenvironment. Successful completion of the above steps, results in (7) successful colonization of a distant organ (78).

Currently, different models of how metastasis exactly occurs are discussed (78). Metastasis itself is a very inefficient process, with only about 0.02% of intravenously (i.v.) injected cells being able to form growing lung metastases (ignoring the percentage of cells capable of leaving the tumor bed) (79). This inefficiency, selects for circulating tumor cells (CTCs) and metastases that possess very specific genetic profiles (80). A process called “self-seeding”, where CTCs return to the primary tumor bed, to promote tumor progression (81). In summary, CTCs (and metastases) experience a sequence of selective processes and acquire a different genetic make-up compared to the primary tumor. Hence, systemic disease responds differently to the treatment regimens, by evaluating the therapy impact on

solely primary tumors in preclinical models. Consequently, the need for the use of sophisticated metastatic models in preclinical research to address the specific make-up of metastases is required.

5.3.3.1 Osteosarcoma metastasis

Not only for cancer in general, also in osteosarcoma metastases are the leading cause of osteosarcoma-related death. At presentation, assessment of potentially metastatic disease is of outermost importance because presence of metastasis is associated with the worst survival prognosis in osteosarcoma (1). Already at diagnosis, between 20 and 25% of the patients have detectable metastases (82). Despite surgical removal of the tumor, approximately 80% of patients with diagnosed “localized disease” will develop macroscopic metastatic disease after surgery (12). Therefore, an equal amount of patients is assumed to have non-detectable microscopic metastases at time of diagnosis (29). Most frequently, osteosarcoma cells colonize the lungs of patients (74% of metastasized cases) and less frequently the bones of patients (10% of metastasized cases) (83). In osteosarcoma, metastasis occurs via haematogenous spread rather than lymphatic spread (84). These circulating tumor cells (CTCs) have a strong prognostic value in other cancers such as breast cancer (85) or Ewing’s sarcoma (86), where overexpression of certain proteins or typical translocations are considered as a defining feature of these cancers and thus, can be used to identify tumor cells in patient blood. In contrast, defining markers for osteosarcomas are missing until now. Nevertheless, a trial in osteosarcoma patients used type I collagen mRNA as a marker for circulating osteosarcoma cells and demonstrated a clinical link between CTCs and detectable osteosarcoma metastases (87). In addition, animal studies highlighted the importance of CTCs and self-seeding for the progression of experimental osteosarcomas (88). Besides CTCs and metastases, recurrences are also prognostic factors for poor survival and indicators for osteosarcoma spread beyond the primary tumor bed. Local recurrences occur in approximately 8-16% of patients and triple the mortality risk, similar to development of metastases (83, 89).

With respect to the molecular biology of osteosarcoma-specific metastasis, less is known compared to carcinoma metastasis because osteosarcomas are rare. Therefore, murine and other animal models mostly contributed to the understanding of mechanisms of osteosarcoma metastasis (84). Osteosarcoma metastases often show large genetic variation compared to their primary tumors, imposing a limitation to treatments that were developed to be effective against primary tumors (90). However, sarcoma cells (including osteosarcoma) do not need to undergo epidermal-to-mesenchymal transition (EMT) due to their mesenchymal origin. Like many cancers, a common requirement for metastasis in sarcoma is the resistance towards anoikis- cell death upon losing contact with the microenvironment (91). Anoikis in sarcomas is overcome by forming homotypic (with other tumor cells) or heterotypic (with platelets or immune cells) cell-cell contacts or overexpressing proteins such as transforming growth factor β (TGF-β) (91). After extravasation, osteosarcoma cells have to avoid the immune system while passing through the blood stream. This is partially enabled through formation of platelet-tumor cell aggregates which disguise the tumor cells and promote arrest of the cells in lung capillaries (in osteosarcoma usually the first organ with small capillaries after leaving the primary tumor bed) (84). Apart from these anatomical restraints, molecular interactions between the primary tumor (e.g. an osteosarcoma) and the target organ (e.g. the lung) were already proposed to play a role in seeding distal organs in the 19th century (92). The molecules priming the future metastatic site are distributed via the blood stream. For instance, a recent study demonstrated a major role of exosomal α6β4 and α6β1 integrins to prime lung tissue for future metastasis in breast cancer (93). Similar to secretion of integrin containing exosomes, secretion of free and exosomal urokinase plasminogen activator (uPA) by osteosarcoma cells may contribute to increasing osteosarcoma metastasis via degradation of extracellular matrix as well as increasing migratory abilities of OS cells (94).

Recent data highlighted the importance of various stromal cells in priming tumor cells for the metastatic process (68). In most cancers, stromal cells such as tumor associated

macrophages (TAMs) are considered to have a metastasis-supporting role (95), whereas in osteosarcoma TAMS were found to be associated with lower numbers of metastases and a good survival prognosis if present at high numbers (96). The direct osteosarcoma inhibitory effects of TAMs are partially achieved after macrophage stimulation with IFN-γ and the secretion of soluble factors, while the precise inhibitory mechanisms are still unknown (97). In contrast to osteosarcoma-suppressing TAMs, other (yet undefined) stromal cells can also increase the propensity of osteosarcoma cells to metastasize. Angiopoietin-like protein 2 signaling was shown to be highly important for enhancing osteosarcoma lung metastasis (98). After demethylation of the promoter via the tumor microenvironment, full length angiopoietin-like protein 2 was expressed in osteosarcoma cells and induced the expression of matrix metalloproteinases (MMPs) that supported intravasation and thus, metastasis of the tumor cells. Although no correlation was found between expression of MMP-9 and metastasis, a metastasis-supporting role of secreted MMPs may exist, possibly via increasing the permeability of the lung endothelial layer, through secretion of systemic MMP-9 (99).

5.4 Current therapy against osteosarcoma

Nowadays, curative treatment of osteosarcoma comprises surgical removal of the primary tumor and chemotherapy using highly cytotoxic drugs. Modern chemotherapy backbones consist of doxorubicin (DOX, also known under the trade name Adriamycin) and cisplatin (CDDP) together with or without methotrexate (100). This chemotherapy backbone (**m**ethotrexate, **A**driamycin, cis**p**latin; MAP) was fine-tuned during the last three decades and increased five-year survival rates to approximately 60-70% (1). In some occasions, ifosfamide or etoposide are added, yet without showing additional benefits over effective MAP-combinations (101-103). Despite the controversy in the use of neoadjuvant chemotherapy, it has been shown to have considerable prognostic value (good response versus poor response) and has therefore replaced solely adjuvant chemotherapy (104). In German-speaking countries, the Salzer-Kuntschik scheme (Table 2) is commonly used to

categorize the histologic response of osteosarcomas in order to evaluate success of neoadjuvant chemotherapy (105). In addition to the prognostic value of histologic response, neoadjuvant chemotherapy reduces tumor volumes prior to surgical resection and thus, facilitates limb sparing procedures rather than amputation to remove the primary tumor (106). The probability to perform limb salvage, however, is higher after a good histologic response was achieved in order to fully remove the tumor while minimizing the risk of local recurrences (107, 108).

Table 1. Definition of histologic response according to Salzer-Kuntschik.

Grade of regression	**Definition**	**Overall**
Grade 1	No vital tumor cells	**Good response**
Grade 2	Individual viable tumor cells or clusters of cells(< 0.5 cm)	
Grade 3	< 10% vital tumor	
Grade 4	10-50% vital tumor	**Poor response**
Grade 5	> 50% vital tumor	
Grade 6	No effect of chemotherapy	

5.4.1 EURAMOS

In order to keep improving therapy for osteosarcoma, larger clinical trials with sufficient statistical power are necessary to compare novel treatments to the current state of the art. However, osteosarcoma is a rare disease, making it difficult to recruit high patient numbers. Therefore, international collaboration as seen in the European and American osteosarcoma study group (EURAMOS)-1 study is required. To date, the EURAMOS-1 has also been the largest clinical study conducted in osteosarcoma, achieved through the international collaboration of four multinational groups: Children's Oncology Group, Cooperative Osteosarcoma Study Group (COSS), European Osteosarcoma Intergroup and Scandinavian

Sarcoma Group (109). Conducting clinical trials within such an international framework will enable future studies to be conducted under accepted standards, at higher speeds due to the large number of available patients and to also have higher statistical power to even detect small improvements in patient survival and compensate for the great heterogeneity of osteosarcomas. The EURAMOS-1 study represents the state of the art of osteosarcoma treatment and nicely illustrates current treatment-related complications as well as optimal survival rates with systemic MAP chemotherapy. One of the major aims of the EURAMOS-1 trial (design shown in Fig. 4) was to evaluate if addition of interferon-α to MAP is beneficial after a good histologic response compared to state of the art MAP chemotherapy only (110).

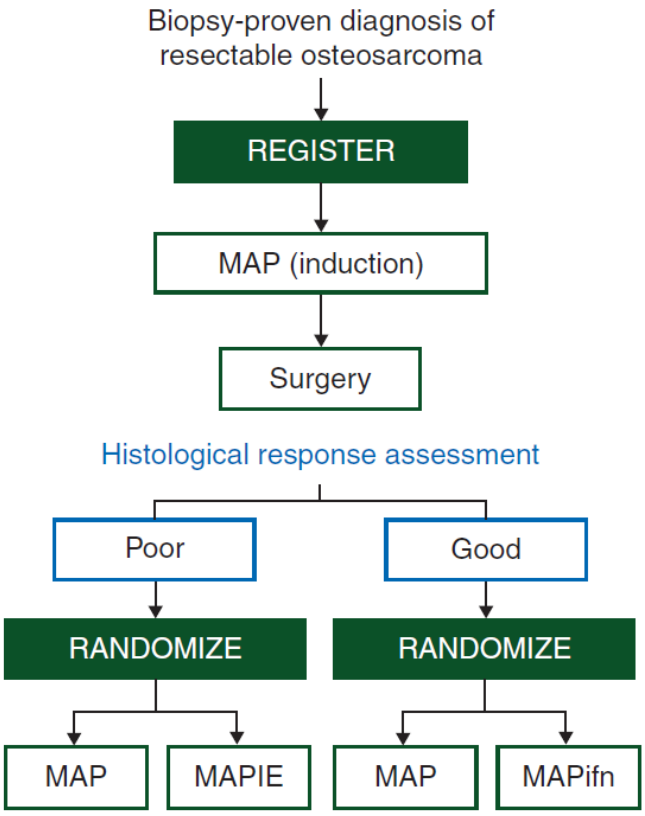


Figure 4. Trial design of the EURAMOS-1 osteosarcoma study. MAPifn: methotrexate, Adriamycin, cisplatin, interferon-α; MAPIE: methotrexate, Adriamycin, cisplatin, ifosfamide, etoposide. Figure adapted from (111) .

At the time of trial initiation in 2005, only a limited amount of data about the effects of interferon-α on osteosarcoma development was available. It was shown *in vitro* that

interferon-α increased the sensitivity of osteosarcoma cells to cytotoxic drugs such as DOX and etoposide, but not towards CDDP, actinomycin D or vinblastine (112). Interferon-α also greatly inhibited the growth of transplanted human osteosarcoma cells in nude mice (113, 114). At the same time, interferon-α treated tumors showed increased amounts of mineralized bone due to the interaction between osteosarcoma and its microenvironment, again shown in athymic mice (115). The *in vivo* data, however, was only derived from immunocompromised mice. Data from a Scandinavian clinical study demonstrated a trend towards better long term survival if higher amounts of interferon-α are given as a single adjuvant therapeutic. The patient cohort that trended towards better survival received larger doses of interferon-α, but also received a different pre-treatment (i.e. absence of radiation) as well as a different treatment after relapse compared to the earlier treated cohort (116). Similarly, a German clinical study tested the use of interferon-β in patients with localized primary osteosarcomas without detecting a significant clinical benefit (117).

Based on the results described above, from *in vitro* studies and studies in nude mice as well as limited clinical information, the EURAMOS-1 trial was frivolously initiated. A total of 2260 patients were recruited, with 716 patients being eligible for evaluation of the interferon-α-treatment. However, evaluation of the current data failed to show a benefit for patient survival after addition of interferon-α (118). Perhaps, earlier addition of interferon-α (i.e. simultaneously to chemotherapy to improve synergy (112)) or the selection of a more appropriate cytokine (e.g. interferon-β has a more potent anti-osteoclastogenic effect (119); interferon-γ stimulates macrophages to inhibit osteosarcoma growth (97)) would have led to improved outcomes of combined immune-chemotherapy against osteosarcoma. In conclusion, an increasing knowledge about the complex interplay between immunotherapy - chemotherapy - osteosarcoma biology is required and sound preclinical models should be evaluated and employed before initiation of future clinical trials in order to assess new regimens with appropriate quality and acceptable standards.

5.4.2 Mechanisms of current chemotherapeutics

Current chemotherapeutics used for osteosarcoma treatment target fast growing cells through interference with cellular replication. Therefore, also non tumor cells are affected by these compounds. CDDP is an inorganic complex, which exerts its toxicity after undergoing a spontaneous stepwise aquation reaction replacing its chlorine atoms (120). Once inside the nucleus, the monoaquated molecule preferably interacts with the N7-site of guanine forming mostly 1,2-intrastrand crosslinks (121). Consequently DNA damage sensors such as MSH2 bind to the CDDP adducts halting the transcription machinery as well as inducing DNA damage signaling. On top of this, the cell cycle will be stopped to allow for nucleotide excision repair, specifically repairing intrastrand crosslinks. Ultimately, all signals seem to be integrated and linked via the tumor suppressor p53. Dependent on the intensities of each signaling pathway, the final fate of the cell is determined. In case of excess DNA damage, the equilibrium is shifted towards irreversible apoptosis.

The anthracycline DOX and etoposide act through inhibition of topoisomerase II by intercalating with the DNA or without, respectively (122). Ultimately these topoisomerase II poisons lead to the formation of covalent protein-DNA complexes and the interference with processes such as DNA replication or transcription, ultimately resulting in apoptosis. Especially in the case of DOX, the topology of DNA is disturbed by evicting histones and increased torsional stress, causing double strand breaks. All of these events impair DNA repair and lead to apoptosis.

Another DNA-crosslinking agent is ifosfamide. After cellular entry, its activated alkyl groups will mostly form DNA interstrand crosslinks (123). These crosslinks lead to the impairment of DNA strand separation during cell division and thus, induce tumor cell death (124). Apart from crosslinking DNA, ifosfamide was also shown to be involved in depleting protective glutathione levels leading to increased DNA alkylation and cytotoxicity (123). In contrast to above described chemotherapeutics, methotrexate does not interact with DNA, it acts via inhibition of dihydrofolate reductase. This enzyme is required to synthesize thymidylate, which is the substrate for thymidine production and hence, DNA synthesis (125). Newer

studies also demonstrated the interference of methotrexate with other cellular enzymes involved in energy production and redox balancing depleting NADPH levels.

5.4.3 Side effects associated with current chemotherapeutics

Although chemotherapy strongly increased overall survival, administration of MAP-chemotherapy is limited by drug-specific toxicities leading, in the worst case, to patient death. Hematological toxicities are commonly observed with standard multidrug osteosarcoma chemotherapy, e.g. neutropenia (52-83% of patients), thrombocytopenia (28-50% of patients) and associated infections (58% of patients) (111, 126, 127). In addition to increased osteosarcoma patient survival rates, modern multidrug-chemotherapy also leads to the development of secondary neoplasms. Even years after completion of chemotherapy, secondary malignancies such as leukemia or breast cancer are observed in 1-9% of osteosarcoma patients (128-131).

Some effects can be specifically ascribed to specific compounds of modern chemotherapy regimens. For instance, cardiac toxicity is associated with the administration of DOX (132). Ototoxicity and associated hearing loss is related to administration of CDDP and can be found in up to 11% of patients (133). Furthermore, renal failure is linked to administration of CDDP as a consequence of CDDP uptake by the proximal tubules and resulting tubular cell death (134, 135). However, advances in understanding the mechanism of nephrotoxicity and improved diuresis reduced the rates of cisplatin-specific renal failure under current clinical standards (111). CDDP was also shown to cause infertility in a dose dependent way in male patients (132). Acute and transient neurotoxicity can be observed after high dose methotrexate or ifosfamide (132). Similar to CDDP, high doses of methotrexate are linked to renal dysfunction in about 2% of osteosarcoma patients, due to the accumulation of methotrexate and its degradation products in the kidney (136). Altogether, systemic MAP chemotherapy and its management is an effective, yet aggressive treatment for patients and its use is limited by the chemotherapy-associated risks and patient discomfort.

In order to improve therapy efficacy without worsening side effects, new strategies are necessary. Approaches such as improved delivery of conventional drugs (achieved through e.g. targeting, liposomal packaging or local application) or the use of novel compounds are required. Ideally, such novel compounds have improved efficacy while inducing less side effects compared to currently used drugs and can therefore replace these drugs. Additionally, the targeted application of novel compounds, combined with standard, already effective drugs at equal or lower concentrations may improve the overall outcome. Some of these strategies to improve osteosarcoma treatment and their corresponding advantages are discussed in the following sections.

5.4.4 Intraarterial chemotherapy

Instead of intravenous (i.v.) drug administrations, local drug administrations via the tumor feeding artery present a more attractive alternative to common systemic administrations. First, such intraarterial (i.a.) drug applications are believed to achieve greater anti-tumor efficacy compared to systemic administrations, due to a first-pass effect by the tumor and subsequently higher intratumoral drug concentrations. At the same time, the resulting systemic drug concentrations will not be significantly reduced, if i.a. infused, and hence, no loss of systemic potency (= the ability to suppress metastases/ systemic disease) is present. Based on these favorable assumptions, potential benefits of i.a. administration of chemotherapeutics were first evaluated by Klopp et al. in 1950 (137). Tests using various animal models revealed a strong local effect of i.a. drug injections while the remaining tissues were spared from drug-induced damage.

With respect to osteosarcoma, the first i.a. drug administrations were performed in the early 1980s (138-140). Studies by Jaffe *et al.* and Stewart *et al.* confirmed a higher bioavailability of for instance CDDP after i.a. infusion (139, 141, 142), potentially leading to superior histologic response rates and superior survival rates with i.a. CDDP (142-144). Despite early trials of using i.a. DOX (145), among the standard drugs against osteosarcoma only CDDP continued to be infused i.a.. Using i.a. CDDP, Wilkins *et al.* achieved good histologic

responses in 87% of patients with localized osteosarcomas. In terms of good response rates, these results compare favorably to other clinical studies investigating localized osteosarcoma in similar patient cohorts treated with i.v. CDDP and i.v. doxorubicin (maximum percentage of patients with good response: 41%) (146), even with the use of a three/ four-drug regimen comprising methotrexate (maximum percentage of patients with good histologic response: 71%) (10, 103, 118, 127, 146, 147). Also in a directly comparative study, i.a. CDDP showed superior histologic response rates compared to i.v. CDDP as part of a three-drug regimen (148).

In compliance with the superior histologic response rates, studies from the St. Luke's Medical Center achieved 10-year survival rates of 82% - 93% using i.v. doxorubicin and i.a. CDDP only (143, 149). These survival rates compare favorably to other studies with maximum 10-year survival rates of, at best, 53- 64% with an i.v. two-drug regimen (35, 146) or up to 70% with an i.v. three/ four-drug regimen (4, 150). Notably, the improved survival rates achieved at the St. Luke's Medical Center seem to be linked to high response rates due to the use of the two most active compounds in osteosarcoma treatment (CDDP and DOX) at high (local) doses. Furthermore, high histologic response rates were well reflected by the arteriographic responses (sensitivity of 98%). Therefore, surgery was performed as soon as an arteriographic response was achieved or the defined maximum number of courses was administered. The so achieved longitudinal monitoring of neoadjuvant chemotherapy outcome avoided unnecessary administration of further courses of chemotherapy, highlighting the general importance of longitudinal therapy response monitoring.

Despite these positive associations with i.a. CDDP, a clinical trial evaluating tumor response according to the route of drug administration was not able to demonstrate unambiguous results in osteosarcoma patients (151). Notably however, patient survival was not evaluated in the course of this study. In another study, superior tumor responses with i.a. CDDP were only seen in the context of a three-drug regimen, whereas i.a. CDDP as part of a four-drug regimen did not yield significantly better responses compared to i.v. CDDP as part of the

same regimens (103). These studies demonstrate the difficulty of evaluating the “true” efficacy of i.a. CDDP due to confounding factors such as administration of combinations of chemotherapeutics, tumor heterogeneity side effect management as well as interinstitutional differences, parameters inherently present in any clinical study environment.

5.4.4.1 Side effects of i.a. CDDP

Besides improved tumor response and survival rates of osteosarcoma patients, i.a. CDDP also generates a different spectrum of side effects compared to equivalent doses of i.v. chemotherapy. In a study comparing i.v. and i.a. application of CDDP in head and neck cancer, a reduction of nephrotoxicity was observed, but also a simultaneous increase of neurological toxicities (152). In another study, again in head and neck cancer, increased rates of cerebral infarction were observed after i.a. chemotherapy, while frequencies of systemic toxicities such as mucosal toxicity or febril neutropenia were not different (153). Similarly, no differences in systemic toxicities were observed between i.a. versus i.v. chemotherapy in glioblastoma (154). In a study comparing i.v. and i.a. chemotherapy in metastatic brain tumors, myelosuppression was worse in the i.v. treatment arm, while other systemic toxicities were similar (155). In contrast, local toxicities were only observed with i.a. chemotherapy.

When considering studies with osteosarcoma patients only, no differences in nephrotoxicity were detected. Similarly, no significant differences in hematological toxicities dependent on the route of administration of CDDP as part of a three-drug regimen were detected in a study performing a direct comparison of i.v. versus i.a. CDDP (103). Furthermore, i.a. CDDP specifically affected the skin close to the infusion site by inducing local swelling and skin necrosis in 6-8% of the patients (103, 151). Myocutaneous inflammation, another i.a. CDDP specific side effect, was observed in 6% of the patients (149). Other frequently reported toxicities observed with i.a. CDDP and simultaneous administration of common chemotherapeutics are mostly similar to systemic chemotherapy only; for instance: mucositis: 27% (with i.a. CDDP) versus 27%-common (with i.v. CDDP); neutropenia:

infrequent versus 58-89%; death due to chemotherapy: 0% versus 0.13-2.8%; leukopenia: 2% versus 79%; thrombocytopenia: 7% versus 52% (103, 104, 108, 111, 143, 149). Although some chemotherapy-related side effects such as death, thrombocytopenia or leukopenia might seem to be reduced with i.a. chemotherapy in osteosarcoma, a direct comparison as presented above is difficult because most parameters were not directly compared within the same study. Thus, interinstitutional differences such as treatment regimens, side effect management or different toxicological grading schemes need to be considered.

5.4.4.2 I.a. chemotherapy in pre-clinical models

Due to the rarity of osteosarcoma, patient numbers are generally low. To improve the understanding of osteosarcoma without having high numbers of patient samples available, researchers often employ animal models to test novel therapeutics or diagnostics. Similarly, i.a. drug administrations were assessed using preclinical animal models of osteosarcoma and other cancers.

Similar to humans, dogs develop osteosarcomas spontaneously, yet at a ten times higher frequency (156). The spontaneous occurrences as well as the resemblance of the human disease in terms of sites of metastasis, sites of primary tumor growth and identical histology of the disease render canine osteosarcomas a valuable “clinical intermediary” for the study of the human disease (157). Based on the characteristics of canine osteosarcomas, Powers *et al.* conducted a study comparing i.v. and i.a. CDDP in dogs bearing spontaneous osteosarcomas (158). Although no obvious difference was found with respect to time to metastasis, the study showed superior histologic responses when CDDP was infused via the tumor feeding artery (49% mean percentage of necrosis) compared to i.v. CDDP (23.8%) infusions. In line with Powers *et al.*, a study of spontaneous head and neck squamous cell carcinomas in sheep employed CDDP as a reference drug and demonstrated objective histologic responses in 73% and 27% for i.a. CDDP infusions and i.v. CDDP infusions, respectively (159).

Also studies of other cancers pointed at an increased tumor perfusion of radiolabeled drug using i.a. infusions (160). In addition, they also explained a decrease in radioactivity of the tumor periphery as well as necrotic areas by a reduced local blood flow. Hence, monitoring of the tumor blood flow is desired to estimate the efficacy of i.a. chemotherapy, as also clinically demonstrated by Wilkins *et al.* (described above (143)). Similarly, Takeda *et al.* showed improved tumor control of i.a. chemotherapy over i.v. chemotherapy in a transplant model of rat brain tumors (161). To also prove validity in human glioma xenografts, an athymic rat model was applied by Schuster *et al.* (162). In addition to superior glioma control, i.a. chemotherapy also significantly increased survival of the rats, when compared to i.v. administrations of a similar or even a two-fold higher dose.

5.4.4.3 Conclusions

In summary, preclinical and clinical studies evaluating i.a. administrations of chemotherapeutics have demonstrated the feasibility of such. Especially in osteosarcoma of the extremities, the use of i.a. chemotherapy through the tumor-feeding artery seems to be advantageous, due to higher drug concentrations within the tumor, a similar if not an improved spectrum of side effects compared with systemic chemotherapy and the avoidance of severe local side effects such as seen after i.a. chemotherapy against malignancies of the brain. Despite proof for improved histologic responses and indications for improved survival rates, the broad use of i.a. chemotherapy in clinical settings has ceased during the last two decades. Mostly reasons such as higher demand on technical requirements, the standard use of multi-agent chemotherapy (103) or the focus on a systemic effect of chemotherapy rather than local control (151) led to the discontinuation of evaluating i.a. administrations of chemotherapeutics. Opposing these assumptions, a better histologic response after i.a. chemotherapy might compromise the remaining primary tumor's ability to seed lung metastases and thus, impact patient survival in the long run. In conclusion, i.a. administration of cytotoxic chemotherapy may be considered for patient treatment as long as there are no

osteosarcoma-specific drugs available in order to improve local tumor control and reduce severe systemic side effects.

5.4.5 Immunotherapy

The immune system (both innate and adaptive) imposes a constant selection pressure on developing cancers. At all stages of cancer progression, osteosarcoma cells have to constantly avoid detection and destruction by the cells of the immune system. Due to the promising long-lasting anti-cancer effects, immunotherapy has already sparked interest in the 19th century, most prominently through the work of William B Coley (163). Back then, Coley produced a mixture of heat-inactivated gram-negative as well as gram-positive bacteria ("Coley's Toxins") that induced a potent infection leading to exceptional regressions of tumors, especially of soft tissue sarcomas. These early successes were most often observed with sarcomas, with about 50% showing long lasting responses (i.e. patients rendered free of disease >5 years). Despite the observed toxicities, these "artificial infections" also showed the huge potential of immunotherapy in achieving long lasting cures. Although inoperable bone cancers showed a great diversity of responses (ranging from poor to good responses) to Coley's Toxins (163), more recent studies demonstrated evidence in favor for the use of immunotherapy against osteosarcoma. Two studies investigating spontaneous osteosarcomas in dogs discovered prolonged survival times if the dogs suffered from a postoperative infection or if they were treated with an inflammation-inducing compound (164, 165).

One of the latest examples that demonstrated the immune system's power is the use of liposomal muramyl tripeptide phosphatidylethanolamine (L-MTP-PE) against human osteosarcoma (166). L-MTP-PE was already approved by the European Medicines Agency (EMA) for use against nonmetastatic osteosarcoma and is thought to act via activation of macrophages and monocytes (167). It was also shown to elevate TNF-α as well as IL-6 plasma levels shortly after administration of L-MTP-PE, in turn causing chills and fever (168). Already in the late 1980s, L-MTP-PE was tested in canine osteosarcoma patients and

achieved outstanding effects by almost tripling the median survival time of dogs compared to a nontreated control cohort (169). Most importantly, administration of L-MTP-PE led to a significant improvement in overall survival if administered with standard chemotherapy (from 70% using standard chemotherapy to 78% with the addition of L-MTP-PE), the largest, significant improvement in patient survival in a randomized phase III trial of osteosarcoma since the introduction of multi-drug chemotherapy (170). Other support for the applicability of immunotherapy against human osteosarcoma is the observation that osteosarcoma patients have an improved survival rate after obtaining a postoperative infection compared to non-infected ones (34). In addition, the early recovery of lymphocytes (37), a higher number of pre-surgical lymphocytes (171) as well as a higher ratio of CD8+/FOX3P+ lymphocytes (172) were shown to be prognostic for improved survival of osteosarcoma patients.

These studies point to a benefit for osteosarcoma patients through immunotherapy and, in light of the plateaued patient survival with the use of chemotherapeutics, support further studies evaluating the full potential of immunotherapeutic approaches against osteosarcoma.

5.4.5.1 TNF-α: general functions and role in cancer therapy

Until 1975 endotoxin was thought to cause hemorrhagic necrosis of tumors. However, Carswell *et al.* identified the active ingredient that caused tumor necrosis in sera of *bacillus* sp. infected and endotoxin treated mice and termed it “Tumor necrosis factor”(TNF) (173). More importantly, they also discovered that TNF is actually produced by the host and not contained in the endotoxin itself. Nowadays, it is known that members of the TNF-superfamily are active in a trimeric form and can be found either soluble or bound to the cell surface (174). TNFs are very potent pro-inflammatory cytokines (stimulate T and B lymphocytes, as well as antigen presenting cells (APC), e.g. dendritic cells) explaining the success of anti-TNF therapy as treatment against various autoimmune diseases (e.g. rheumatoid arthritis (175), Crohn’s disease (176, 177)).

One of the most prominent members of the TNF superfamily is the type II transmembrane protein TNF-α which is mostly produced by macrophages and T lymphocytes (178, 179).

After cutting of the membrane bound TNF-α via TNF-α converting enzyme (TACE), soluble TNF-α will bind to TNF receptor 1 (TNFR1) and mostly induce pro-inflammatory as well as the programmed cell death pathways (179, 180). In contrast, transmembrane TNF-α is superior to its soluble form in binding to TNFR2 that is mainly expressed by immune cells and endothelial cells and thus, evoking signaling cascades leading to T cell activation, thymocyte proliferation and granulocyte/macrophage colony-stimulating factor (G/ M-CSF) production (181). Therefore, TNF-α signaling via TNFR1 is usually associated with cytotoxic and pro-inflammatory signaling whereas TNF-α signaling via TNFR2 is rather associated with cell migration, activation and proliferation (179). Furthermore, systemic TNF-α signaling during inflammation leads to vasodilation (182), disruption of the integrity of the endothelial monolayer (183), edema formation and the adhesion of leukocytes (184). On the one hand, the strong pro-inflammatory capability of TNF-α render it an interesting candidate for anti-cancer (immuno)therapy. On the other hand, approximately 15% of cancers are attributable to infections and underlying inflammations (185). Besides forming a cancerogenic inflammatory environment, chronic inflammation also commonly induces bone resorption (186). Especially in the case of osteotropic malignancies such as osteosarcoma, chronic inflammation or immunotherapy must be well controlled, because of its potential in promoting the vicious cycle of bone remodeling and thus, osteosarcoma progression (187). In general, local concentrations of TNF-α are critical. Locally high concentrations of TNF-α induce tumor necrosis and locally low and chronic concentrations promote tumorigenesis (188).

Experiments in the 1980s demonstrated promising anti-tumor effects of TNF-α against experimental sarcomas (189). However, in order to cause complete tumor necrosis, TNF-α was dependent on its ability to destroy the tumor vasculature (190) and the presence of tumor-specific immunity (191, 192). These results are further supported through *in vitro* tests where TNF-α required an additional stressor such as actinomycin-D to induce broad tumor cell death (188). Despite the impressive anti-tumor effects of TNF-α against cancers, its systemic use is limited by severe toxicities- similar to the administration of Coley's Toxins.

TNF-α can induce shock, tissue injury, gastrointestinal necrosis, acute renal tubular necrosis, among others, even at TNF-α levels which could be endogenously produced by the host (193).

In order to overcome these important limitations, researchers started to investigate the potential of isolated limb perfusions (194, 195), local delivery of the TNF-α gene via intratumoral injections (196), modification of the TNF-α protein to be less toxic (197) or by adding a targeting moiety (198). To date, isolated limb perfusion (ILP) of TNF-α and melphalan demonstrated feasibility and indicated clinical success in treatment of advanced soft tissue sarcomas and melanomas (195, 199, 200). A feasibility study, testing ILP with TNF-α and CDDP in dogs with spontaneous osteosarcomas, was marked by a high mortality rate (201). In humans, treatment of osteosarcoma using ILP with TNF-α, melphalan and interferon-γ showed high percentage of tumor necrosis despite absence of significant tumor shrinkage (202). In addition, the remaining tumors formed a thick collagen-I capsule which facilitated surgical removal of the primary tumor. However, similar to the dog study described above, mortality of ILP with TNF-α in human osteosarcoma patients was high (62% died within 1 year) (203). Thus, the use of ILP with TNF-α as a treatment strategy remains to be only used by single specialized institutions. Likewise, intratumoral injections are not commonly used in clinics as a route of drug administration due to the relatively uncontrolled delivery (similar to intraperitoneal (i.p.) injections). In conclusion, the only direct means of ultimately administering TNF-α to patients without evoking severe sepsis is the modification of the biologically active compound, for instance through addition of a targeting moiety.

5.4.5.1.1 TNFα in osteosarcoma

Preclinical studies mostly reported conflicting results about the efficacy of TNF-α against osteosarcomas. For instance, experimental studies demonstrated tumor-promoting abilities of TNF-α addition, reflected by an increased metastatic potential (204, 205), being non-responsive to TNF-α induced cell death or maintaining osteosarcoma cells in an undifferentiated state (206). In a rat *in vivo* model, experimental osteosarcomas showed no

response upon TNF-α ILP (with melphalan) due to a claimed lack of immunogenicity and a relatively low microvessel density (207). In contrast, the same osteosarcoma cell line showed growth retardation if subcutaneously (s.c.) implanted and treated with ILP of TNF-α and DOX, likely, because of increased intratumoral DOX concentrations (208). Clear immunotherapeutic potential and involvement of TNF-α in the treatment of osteosarcomas was demonstrated in a study using microwave ablated osteosarcoma cells, used as a vaccine to inhibit tumor growth in mice (209). At the same time, microwave ablation *in situ* actively protected rats from further tumor growth due to rejection by the immune system after TNF-α stimulation. These conflicting results might be explained by the fact that these experimental models used artificial microenvironments (i.e. *in vitro* or s.c./ i.p. xenografts), whereas it would be more clinically relevant to assess the effect in a bony microenvironment. In summary, systemic TNF-α or ILP with TNF-α do not seem to be appropriate for human use without further adaptations.

5.4.5.1.2 Targeted formulations of TNF-α

The actual anti-tumor effect of TNF-α is exerted through indirect effects (210) such as disturbance of the tumor vasculature integrity (183) or stimulation through its immune activating properties (191, 211). The tumor or host derived endothelial cells and precursors can be destroyed through the action of TNF-α (191, 199) or become activated to induce blood coagulation (212). These desired effects of TNF-α on the tumor vasculature are further amplified through addition of targeting moieties (e.g. NGR (a common peptid motif on neovasculature), F8 (targets the EDA domain of fibronectin), L19 (targets the EDB domain of fibronectin), TCP-1 (a peptide motif targeting vasculature of colorectal cancers)), with the aim of minimizing its systemic toxicity through tumor-/ neovasculature targeted delivery (198, 213-215). TNF-α itself also increases the permeability of blood vessels or increases their stability dependent on its local concentration (183, 216). Considering the effects of TNF-α on the tumor vasculature, co-administration of TNF-α with chemotherapeutics has the potential to increase the intratumoral concentration of drugs (208) and consequently, enhance their

potency (189, 217). Most importantly, targeting of TNF-α allowed the systemic application of the cytokine at efficacious concentrations, without causing systemic toxicity (213).

5.4.5.2 Immunostimulatory characteristics of conventional chemotherapy

Against the assumption of mostly direct cytotoxic effects of conventional chemotherapy on tumor cells without an obvious link to the host's immune system, more and more evidence accumulates for the importance of such a connection. In fact, the presence and composition of immune cells in the tumor bed strongly influences pathologic tumor responses as well as cancer patient survival (218-220). Likewise, the absence of lymphocytes in the blood has a negative influence on patient survival. As demonstrated in soft tissue sarcoma, breast cancer and lymphoma, lymphopenia (<1000 lymphocytes/µl) is a strong prognostic factor for poor overall survival (221). Initially, chemotherapeutics were selected and tested based on their direct tumoricidal effects. However, most of these drugs (>50%) were recently shown to have at least some influence on the host immune system, even below tumoricidal concentrations (e.g. a concentration dependent effect on maturation/ cell death of DCs (222)). Especially conventional and already successful chemotherapeutics such as anthracyclines (e.g. DOX) or oxaliplatin (not CDDP), also act as potent immunotherapeutics. Anthracyclines were shown to naturally evoke an immunogenic cell death (ICD) response dependent on endoplasmatic reticulum (ER) stress signaling (223). This ICD is dependent on the action of TLR4+ dendritic cells (DC) and cytotoxic CD8+ T lymphocytes (224). In contrast to DOX, CDDP or mitomycin C by themselves are not capable of inducing ICD (225). However, induction of ER stress signals such as translocation of calreticulin from the lumen of the ER to the cell surface (e.g. by digoxin treatment) in combination with CDDP treatment is capable of inducing ICD (226). Expression of calreticulin (or HSP90) on the cell surface of dying tumor cells leads to antigen engulfment by DC and maturation of DC for proper tumor antigen presentation and mounting tumor-specific immune responses via CTLs (227, 228).

Other than altering the host's immune system, immunotherapy (e.g. TNF-α) and chemotherapy can be combined to evoke beneficial microenvironmental changes. For

instance, TNF-α was shown to synergistically cause increased necrosis in sarcomas as well as osteosarcoma animal models if applied together with DOX or other chemotherapeutics (189, 208, 229, 230), which can be explained by the disruption of the endothelial layer due to co-administration of TNF-α, leading to enhanced vascular leakage and increased intratumoral DOX concentrations (183, 208, 230).

5.4.5.3 Conclusions

In summary, improving immunotherapy of osteosarcoma has the potential to increase patient survival. However, the main requirement for future therapies to be successful is a better understanding of the interplay between the immune system and the microenvironment of bone. For instance, similar to the “malignant” vicious cycle (see Fig. 1), an inflammation-induced vicious cycle (i.e. by T lymphocytes) can also cause bone resorption. Not only RANKL from T lymphocytes, but also expression of TNF-α due to chronic inflammatory conditions (e.g. rheumatoid arthritis) leads to activation of osteoclast activity and hence, bone resorption (231). Ultimately, the precise role (i.e. inhibitory or activating) of TNF-α on osteoblast activity depends on the local tissue concentration as well as the expression state of the osteoblast (232). These examples nicely demonstrate the importance of understanding the role of inflammatory signaling in the context of bone remodeling. Last but not least, the effects of conventional chemotherapeutics need to be discovered with respect to their immune effects. Knowing the concentrations at which chemotherapeutics achieve optimal immunostimulatory effects will allow the tailored application of backbone chemotherapy to optimally influence the host immune system and consequently improve osteosarcoma treatment.

6 Aims of the thesis

Chemotherapy is the mainstay of osteosarcoma therapy besides surgical removal of the tumor. In contrast to surgery, chemotherapy not only impacts the primary tumor or large, resectable metastases, it also hinders the growth or even eradicates micrometastatic disease. Already four decades ago, the introduction of chemotherapy led to the largest improvement of survival rates in osteosarcoma. In order to build upon the success of already established chemotherapeutics, the major goal of this work is to (re)assess modern chemotherapy from a today's point of view. Specifically, is there a way to optimize current combinatorial practices to improve their overall therapeutic efficacy?

In order to answer these questions, our work is divided into three sub aims:

1- Effect of standard chemotherapy on biomarkers in human osteosarcoma tissue. The expression of biomarkers associated with therapy outcome may help to stratify patients, ideally at diagnosis, to receive an adapted chemotherapy schedule according to the aggressiveness of the disease. This helps to avoid administration of chemotherapy to patients that might not benefit based on their biomarker profile and thus, spares them from the associated side effects. In order to obtain the necessary knowledge about potential biomarkers of a rare cancer such as osteosarcoma, an optimal use of the available tumor tissue is required. To achieve this goal, we investigated the impact of conventional chemotherapy on the expression of specific biomarkers (e.g. tumor suppressors) by using a tissue microarray to study the expression of selected biomarkers in human osteosarcoma samples prior to and after chemotherapy.

2- Increasing the anti-tumor efficacy of conventional chemotherapy without decreasing its systemic potency. Intraarterial drug administrations via the tumor feeding artery allow for higher regional drug concentrations. It is believed, that i.a. CDDP is superior to i.v. CDDP in terms of tumor control. However, clinical studies so far could not show a true benefit of i.a. over i.v. CDDP administration. Furthermore, little is known about the impact of i.a. CDDP on the development of pulmonary metastasis. Therefore, we used a mouse model

of experimental metastatic osteosarcoma to compare the effects of i.a. with i.v. CDDP-based chemotherapy on osteosarcoma development, including its associated toxicities.

3- Combining conventional chemotherapy with a novel immunotherapeutic drug against metastatic osteosarcoma. The use of conventional chemotherapy improved overall patient survival compared to surgery alone. Five-year survival rates plateaued at 60-70% for localized disease, whereas survival rates for metastatic osteosarcomas remain at a low 20%, despite the use of highly cytotoxic chemotherapy. Therefore, novel approaches to target metastatic disease are urgently needed. Based on initial preclinical successes in soft tissue sarcomas, we believe that TNF-α also has the potential to treat osteosarcomas. To this end, we evaluated the effect of a vasculature-targeted version of TNF-α (F8-TNF) using a syngeneic metastatic mouse model. First, drug efficacy was evaluated in the presence of a primary tumor. Next, efficacy of F8-TNF, alone or with DOX, was evaluated in a syngeneic mouse model of systemic osteosarcoma.

7 Results

7.1 Prognostic value of tumor suppressors in osteosarcoma before and after neoadjuvant chemotherapy

Bernhard Robl[1], Chantal Pauli[2], Sander Martijn Botter[1], Beata Bode[2] and Bruno Fuchs[1,§]

1 Laboratory for Orthopedic Research, Department of Orthopedics, Balgrist University Hospital, Zurich, Switzerland

2 Institute of Surgical Pathology, University Hospital Zurich, Zurich, Switzerland

Published in “BMC Cancer” on the 9th of May 2015.

Citation: Robl *et al*.. BMC Cancer. 2015 May 9;15:379.

doi: 10.1186/s12885-015-1397-4.

Submitter’s contributions to the manuscript:

Conception and design; Development of methodology; Acquisition of data; Analysis and interpretation of data; Writing of the manuscript; Administrative and material support (i.e., reporting and organizing data);

Robl et al. BMC Cancer (2015) 15:379
DOI 10.1186/s12885-015-1397-4

BMC Cancer

RESEARCH ARTICLE Open Access

Prognostic value of tumor suppressors in osteosarcoma before and after neoadjuvant chemotherapy

Bernhard Robl[1], Chantal Pauli[2], Sander Martijn Botter[1], Beata Bode-Lesniewska[2] and Bruno Fuchs[1*]

Abstract

Background: Primary bone cancers are among the deadliest cancer types in adolescents, with osteosarcomas being the most prevalent form. Osteosarcomas are commonly treated with multi-drug neoadjuvant chemotherapy and therapy success as well as patient survival is affected by the presence of tumor suppressors. In order to assess the prognostic value of tumor-suppressive biomarkers, primary osteosarcoma tissues were analyzed prior to and after neoadjuvant chemotherapy.

Methods: We constructed a tissue microarray from high grade osteosarcoma samples, consisting of 48 chemotherapy naïve biopsies (BXs) and 47 tumor resections (RXs) after neoadjuvant chemotherapy. We performed immunohistochemical stainings of P53, P16, maspin, PTEN, BMI1 and Ki67, characterized the subcellular localization and related staining outcome with chemotherapy response and overall survival. Binary logistic regression analysis was used to analyze chemotherapy response and Kaplan-Meier-analysis as well as the Cox proportional hazards model was applied for analysis of patient survival.

Results: No significant associations between biomarker expression in BXs and patient survival or chemotherapy response were detected. In univariate analysis, positive immunohistochemistry of P53 ($P = 0.008$) and P16 (P16; $P = 0.033$) in RXs was significantly associated with poor survival prognosis. In addition, presence of P16 in RXs was associated with poor survival in multivariate regression analysis ($P = 0.003$; $HR = 0.067$) while absence of P16 was associated with good chemotherapy response ($P = 0.004$; $OR = 74.076$). Presence of PTEN on tumor RXs was significantly associated with an improved survival prognosis ($P = 0.022$).

Conclusions: Positive immunohistochemistry (IHC) of P16 and P53 in RXs was indicative for poor overall patient survival whereas positive IHC of PTEN was prognostic for good overall patient survival. In addition, we found that P16 might be a marker of osteosarcoma chemotherapy resistance. Therefore, our study supports the use of tumor RXs to assess the prognostic value of biomarkers.

Keywords: P53, PTEN, P16, Osteosarcoma, Chemotherapy, Tissue Microarray, Tumor Suppressor Genes

Background

Osteosarcoma is the most common malignancy of bone and among the deadliest cancers in adolescents [1, 2]. Osteosarcoma patients are commonly treated with multiagent neoadjuvant chemotherapy, combined with surgery to remove the primary tumor mass and subsequent adjuvant chemotherapy. Introduction of chemotherapy has increased the mean 5-year survival rates of patients with localized disease from 20 % in the early 1970s to above 60 % at present [1, 3]. In contrast, the presence of metastases is a strong prognostic factor for poor survival rates of 30 % or less [4].

Specifically for osteosarcoma, a patient's response to neoadjuvant chemotherapy has a considerable prognostic value and has therefore replaced single adjuvant chemotherapy [5, 6]. To date, necrosis of tumor resections (RXs) above 90 %, although only a crude read out, is still used in clinical practice due to its prognostic

* Correspondence: research@balgrist.ch
[1]Laboratory for Orthopedic Research, Department of Orthopedics, Balgrist University Hospital, Forchstrasse 340, 8008 Zurich, Switzerland
Full list of author information is available at the end of the article

power for patient survival [4, 7]. Current protocols of neoadjuvant chemotherapy for routine use in osteosarcoma are based on combinations of highly cytotoxic drugs such as cisplatin, methotrexate and doxorubicin [8]. Although potent, these drugs are not specific enough and tumor resistance, subsequent disease progression as well as patient death are therefore frequently observed. Consequently, numerous immunohistochemical studies have tried to identify osteosarcoma-biomarkers. For instance, VEGF [9-11], ezrin [12-14], P53 [15], P16 [16], CD44 [17], CXCL12 [18] were evaluated as prognostic factors for survival, whereas immunohistochemical stainings of nuclear P16 [19], CRIP1 [20] and COX-2 [21] were investigated as predictors of chemotherapy response.

However, the above mentioned studies only evaluated chemotherapy naïve biopsies (BXs) of osteosarcomas. Despite the larger amounts of available tissue compared to needle biopsied tissue, fewer studies analyzed RXs because it is thought that no valid prediction about patient survival can be made. Nevertheless, analysis of marker expression in remaining viable tumor tissue after chemotherapy can be investigated similar to assessing the degree of response to chemotherapy [22], and may yield important information about patient prognosis and the impact of chemotherapy in non-responders. Similarly, only a few studies analyzed RXs of osteosarcomas in order to study expression changes of biomarkers prior to and after chemotherapy yet in these studies significant correlations were found between clinicopathological parameters and expression changes of biomarkers such as VEGF [23], MMP-2 [24], ezrin [25] and alkaline phosphatase [26].

Tumor suppressors are thought to have a major impact on the response to chemotherapy [27-30] and hence, patient survival. In this study, we therefore analyzed immunohistochemical stainings in BXs and RXs of four established tumor suppressors (P53, P16, PTEN and maspin) in viable patient-derived tissue before and after neoadjuvant chemotherapy in order to better understand their changes during chemotherapy, and to find out if this change is related to chemotherapy response or survival. Wild-type P53 is a major player in the DNA damage response and initiates cell apoptosis once the extent of DNA damage is beyond repair [31]. Intriguingly, wild-type P53 also has the ability to protect tumors during chemotherapy [27], highlighting the need for a better characterization of P53 as a marker during chemotherapy. P53 is well known to be mutated in high grade osteosarcomas [15, 32] and mutant P53 is often detected by immunohistochemistry (IHC) due to its increased half-life [33], highlighting its potential as a valuable marker for osteosarcoma patient prognosis.

P16 is considered another major tumor suppressor and acts through blocking of cyclin dependent kinase 4 signaling and consequently, cell cycle progression [34]. P16 as a biomarker is less well characterized than P53 in osteosarcoma. Nevertheless, the use of osteosarcoma BXs identified P16 as a sensitive prognostic factor [35] and to be predictive for good chemotherapy response [19]. In contrast to what is inferred from biopsied samples, changes in P16 might protect the tumor cells during chemotherapy by decreasing their proliferation rate [28]. Therefore, it is of importance to not only study the presence of individual tumor suppressors but to also investigate their impact on tumor proliferation (e.g., monitoring of Ki67 [36]). Tumor proliferation measured by Ki67 indices is believed to have strong prognostic value in multiple types of cancer [37-39]. Proliferation is the result of an excess of growth promoting signals such as growth-factor signaling pathways or the inhibition of cell cycle regulators. Thus, proliferation can be altered at various levels, for instance through upregulation of BMI1, causing a deactivation of P16 and hence, an increase in proliferation [40]. At the same time, BMI1 was found to be overexpressed in more than half of chemotherapy naïve osteosarcoma specimens [41]. To date, no significant correlation between clinicopathological parameters and BMI1 expression in osteosarcomas was discovered.

Two additional tumor suppressors, maspin and PTEN, have hardly been studied in the context of osteosarcoma so far. Similar to P16 [34], PTEN controls cell proliferation by regulating cyclin D levels and inhibiting PI3K-Akt signalling [42]. Thus, presence of PTEN in tumor specimens is considered as being prognostic for good patient survival [43, 44]. The precise mechanism of maspin, on the other hand, is still under debate. On the one hand, studies showed a reduced metastatic potential of breast cancer cells [45] or augmented cancer cell death by chemotherapeutic drugs through induction of maspin [29] and, on the other hand, studies demonstrated increased expression of maspin to be an indicator of poor survival [46] or poor chemotherapy response [47]. These controversial results suggest that expression changes of maspin are rather secondary effects caused by similar changes to adjacent and more relevant genes, further supported by a recent study [48].

Drugs used in current chemotherapeutic protocols to treat osteosarcomas generally induce tumor cell death, yet some tumors adapt in order to avoid death. In order to identify patients at risk, we studied the before mentioned biomarkers in primary osteosarcoma tissues before and after chemotherapy. Using immunohistochemistry (IHC), we evaluated associations between the presented biomarkers and clinical parameters such as overall survival, response to chemotherapy, metastasis or proliferation of the primary tumor in order to assess the clinical value of the studied biomarkers.

Methods

Patient samples

This retrospective study was conducted with tumor tissue specimens from a total of 61 patients who were operated between December 1987 and October 2005. The specimens were retrieved from the archive of the Institute of Surgical Pathology of the University Hospital, Zurich, Switzerland. All tissue samples were graded as high grade osteosarcomas according to the current histopathological classification by the World Health Organization [49]. Follow-up was started at first diagnosis of the osteosarcoma and ended at death or with the last clinical record in our hospital database giving a range of 7–210 months with a median follow-up of 85 (BX) and 90 (RX) months. All patients used for survival analysis had a complete clinical record and a follow-up of at least 50 months. Patients receiving complete neoadjuvant chemotherapy according to the formerly used COSS protocols, namely COSS-86, COSS-91 and COSS-96 [50, 51] were retrospectively selected and the corresponding clinical records were reviewed and updated. Tumor response was evaluated based on the grading of tumor necrosis according to the guidelines by Salzer-Kuntschik *et al.* [22]. Patients were termed "responders" if tumor necrosis, based on histopathological analysis, was greater than 90 % after neoadjuvant chemotherapy and "non-responders" if less than 90 % of the tumor was necrotic. Ultimately, panels of 47 chemotherapy naïve biopsies (herein termed BXs), 44 neoadjuvant chemotherapy treated tumor samples (i.e., resections, herein termed RXs) and 11 lung metastasis-derived tissues were analyzed. In a maximum of 31 cases, a matched chemotherapy-naïve BX and neoadjuvant chemotherapy treated RX of the same patient were obtained and used for the analysis of changes of IHC in BXs and RXs derived from the same patient.

Tissue microarray

In this study a tissue microarray (TMA) containing paraffin-embedded primary tumor material (both BXs and RXs as well as lung metastases, see ref [52]) was used to assess marker expression. Based on hematoxylin and eosin stained sections of the tumor, viable tumor cell containing areas were selected for the construction of the TMA. All BX-derived available tissue cores with sufficient numbers of tumor cells were evaluated. For RX derived material, only tissue cores derived from "non-responders" (defined as Salzer-Kuntschik grade 4–6) and "responders" (Salzer-Kuntschik grade 2 and 3) were considered for analysis, due to a lack of viable tissue in grade 1 "complete responders".

Immunohistochemistry and TMA analysis

Immunohistochemistry (IHC) was carried out on 4 μm sections of the TMA. Sections were transferred to an adhesive-coated slide system (Instrumedics, Hackensack, NJ, USA), deparaffinized, and processed with an automated Ventana Benchmark staining system (Ventana Medical Systems Tucson, Arizona, USA). Heat-mediated antigen retrieval was performed with cell conditioner 1 for at least 30 min. Individual sections were probed with the following antibodies: mouse monoclonal anti-$P16^{ink4a}$ (clone 16P04, dilution 1:600; LabVision/Neomarkers, USA), mouse monoclonal anti-P53 (clone DO-7, dilution 1:80; Dako, DNK), mouse monoclonal anti-PTEN (dilution 1:200; clone 28H6; Leica Biosystems/Novocastra, GER), mouse monoclonal anti-maspin (clone G167-70, dilution 1:200, BD Pharmingen, USA), mouse monoclonal anti-BMI1 (clone F6, dilution 1:50; Millipore/Upstate, USA) and mouse monoclonal anti-Ki67 (clone MIB-1, dilution 1:20; Dako, DNK). Visualization of the antibody binding was done by applying the iVIEW DAB Kit (Ventana Medical Systems Tucson, Arizona, USA). Slides were counterstained with hematoxylin. A pathologist (CP) and an instructed scientist (BR) independently analyzed the tissue cores in a blinded fashion, where special attention was given to the subcellular (nuclear or cytoplasmic) localization of the analyzed marker. A consensus grading was formed in case of differences between individual samples. At least two cores per patient sample were analyzed to compensate for tissue heterogeneity. Tissue cores were graded as "negative" (grade 0) if less than 10 % of the tumor cells were stained, as "positive" (grade 1) if between 10 and 50 % of the tumor cells were immunostained with intermediate or high intensity and as "strongly positive" (grade 2) if more than 50 % of the tumor cells were stained with high intensity. In addition, changes of biomarkers following chemotherapy were investigated by comparing the immunohistochemical grades of BXs and RXs of the same patient. These changes were classified as "increase", "no change" or "decrease" of the respective biomarker.

Statistical analysis

Kaplan-Meier curves were used to calculate overall patient survival, which was defined as the time from diagnosis until death or until last follow-up. Log-rank tests were used to assess the statistical difference between groups. Multivariate Cox regression models were used to calculate hazard ratios (HRs) and 95 % confidence intervals (CIs). The clinicopathologic factors patient age, gender, location of primary tumor occurrence and histological subtype of osteosarcoma were included as covariates next to expression of individual biomarkers. Multivariate binary logistic regression models were used to estimate Odds ratios (ORs) as well as 95 % CIs. To determine associations between biomarker expression and other parameters (i.e., proliferation (Ki67 immunostaining); presence of metastasis) Fisher's exact tests were applied. All statistical tests

were 2-sided where $P < 0.05$ was regarded as statistically significant. PASW Statistics 18.0 (IBM Corp., USA) was used for statistical evaluation.

Ethics statement

The design of this retrospective study was assessed and approved by the local ethics committee of the University Hospital Zurich (approval reference number StV 41–2005).

Results

Patient cohort characteristics

As depicted in Table 1, the two patient cohorts used for analyses of BXs or RXs had similar clinicopathological characteristics. In both cohorts, the majority of the patients were male (BX: 60 %, RX: 64 %) and the overall mean age was 18.4 years and 18.0 years in the BX cohort and RX cohort, respectively. Most osteosarcomas were seen in patients aged 10–24 years (BX: 65 %, RX: 66 %). The distribution of histological subtypes such as the predominant osteoblastic type (BX: 71 %, RX: 70 %) or the main sites of primary tumor occurrence such as the tibia/ fibula/ calcaneus (BX: 40 %, RX: 32 %) or the femur (BX: 38, RX: 43 %) were similar in both patient cohorts. A total of 65 % (BX) and 68 % (RX) of patients were alive at the last follow-up resulting in similar five-year survival rates of 65 % (BX) and 68 % (RX). Chemotherapy response (≥90 % tumor necrosis) subsequent to neoadjuvant chemotherapy was found in 54 % (BX) and 49 % (RX) of the patients compared to 46 % (BX) and 51 % (RX) being non-responders (<90 % tumor necrosis). During follow-up, 44 % (BX) and 38 % (RX) of patients developed metastases.

Tumor IHC

Representative examples of positive as well as negative immunohistochemical stainings of BXs are given in Fig. 1. The subcellular localization differed between the analyzed markers: IHC of P53 (Fig. 1b), Maspin (Fig. 1d), Ki67 (Fig. 1e) and BMI1 (Fig. 1f) showed nuclear localization in >90 % of the positively stained BXs. In contrast, PTEN (Fig. 1c) was exclusively found in the cytoplasm. Subcellular localization of P53, Maspin, Ki67, BMI1 and PTEN in RXs was the same as in BXs. IHC of P16 (Fig. 1a) showed equal numbers of "cytoplasmic and nuclear" as well as "cytoplasmic only" (see Additional file 1) P16-positive BXs (52 % versus 48 % of the BX samples, respectively). In P16-positive RXs, "cytoplasmic only" expression of P16 was more frequent than "cytoplasmic and nuclear" localization of P16 (65 % versus 35 % of the RX samples, respectively). Furthermore, all P16-positive osteosarcoma samples had detectable P16 in the cytoplasm of cancer cells, whereas no sample was found with a "nuclear only" subcellular localization of P16.

Table 1 Clinicopathologic characteristics of high-grade osteosarcoma patients and IHC of six biomarkers

Variables	n_{BX}	$\%_{BX}$	n_{RX}	$\%_{RX}$
All high grade osteosarcoma	48	100	47	100
Neoadjuvant chemotherapy	48	100	47	100
Sex				
Female	19	40	17	36
Male	29	60	30	64
Patient age				
<10 years	10	21	9	19
10–24 years	31	65	31	66
>24 years	7	15	7	15
Histological subtype				
Osteoblastic	34	71	33	70
Chondroblastic	4	8	7	15
Fibroblastic	5	10	4	9
Telangiectatic	5	10	3	6
Location				
Tibia / Fibula / Calcaneus	19	40	15	32
Femur	18	38	20	43
Humerus / Ulna	6	13	5	11
Axial	5	10	7	15
Pathologic Response				
Responder	26	54	24	49
Non-Responder	22	46	23	51
Metastasis				
Yes	21	44	18	38
No	27	56	29	62
P16 total ($n_{matchedBX\text{-}RX} = 27$)	44	100	39	100
$P16_{positive}$	25	57	17	44
$P16_{negative}$	19	43	22	56
P53 total ($n_{matchedBX\text{-}RX} = 31$)	47	100	44	100
$P53_{positive}$	9	19	12	27
$P53_{negative}$	38	81	32	73
PTEN total ($n_{matchedBX\text{-}RX} = 10$)	40	100	22	100
$PTEN_{positive}$	25	63	7	32
$PTEN_{negative}$	15	37	15	68
Maspin total ($n_{matchedBX\text{-}RX} = 21$)	39	100	33	100
$Maspin_{positive}$	26	67	10	30
$Maspin_{negative}$	13	33	23	70
Ki67 total ($n_{matchedBX\text{-}RX} = 15$)	43	100	25	100
$Ki67_{positive}$	24	56	8	32
$Ki67_{negative}$	19	44	17	68
BMI1 total ($n_{matchedBX\text{-}RX} = 16$)	42	100	28	100
$BMI1_{positive}$	11	26	2	7
$BMI1_{negative}$	31	74	26	93

BX biopsy, *RX* resection

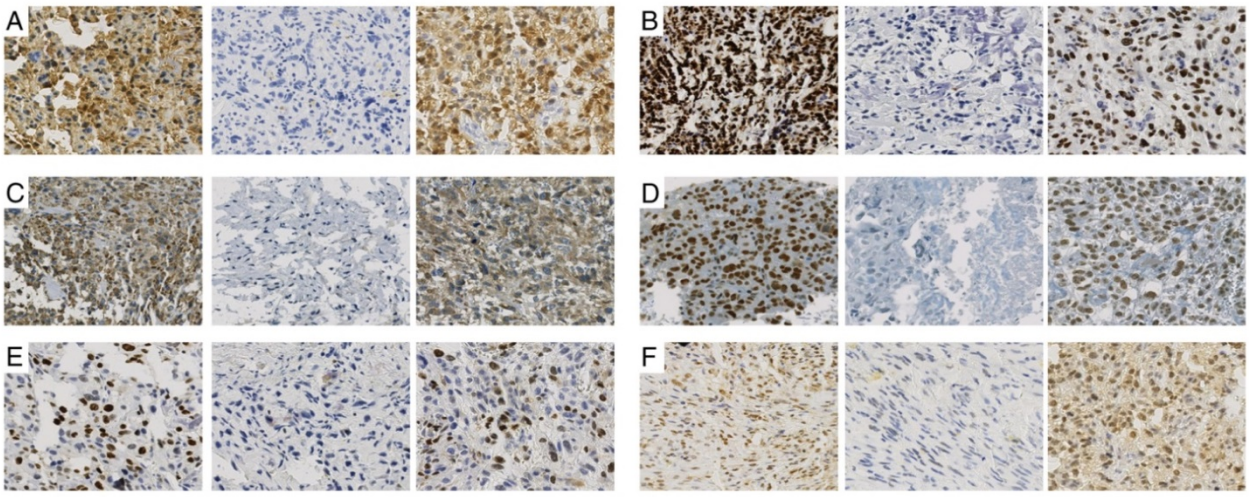


Fig. 1 Representative images of immunohistochemistry of the six analyzed biomarkers. For each part (**a–f**) the same order of samples is shown: left (positive staining), middle (negative staining), right (positive staining of a lung metastasis). **a**, nuclear and cytoplasmic P16 staining. **b**, nuclear P53. **c**, cytoplasmic PTEN. **d**, nuclear maspin. **e**, nuclear Ki67. **f**, nuclear BMI1. Normalized magnification of all images, 40x; Hematoxylin counterstaining

Table 1 also indicates the numbers of samples available for each analyzed marker. In general, a larger number of BXs (n_{BX}: 39–47) was available for IHC compared to specimens derived from RXs (n_{RX}: 22–44). A positive BX staining was most often found for maspin and PTEN, in with approximately two thirds of the samples were positively stained. Staining of P53 and BMI1 was less common, with 19 % and 26 % positive staining, respectively. In over half of the BXs, Ki67 (56 %) and P16 (57 %) could be detected. As depicted in Table 1, chemotherapy decreased the immunohistochemical grade of P16, PTEN, maspin, Ki67 and BMI1, i.e., led to a decreased expression of the marker in the patient samples after chemotherapy. This decrease was most dramatic for BMI1 and maspin, where in relative terms, more than half of the samples lost their marker expression. In contrast, a relative increase of P53-positive samples was observed after chemotherapy (BX, positive: 19 %; RX, positive: 27 %).

Survival analysis

As depicted in Fig. 2. Kaplan-Meier survival analysis of chemotherapy-naïve BXs of high grade osteosarcoma patients yielded no significant differences in overall survival for the various biomarkers, although for P53 a trend was observed for worse survival in case of presence of nuclear P53 ($P = 0.083$; Fig. 2b). In contrast, the analysis of patient RXs yielded significant differences in overall survival as illustrated in Fig. 3. Positive expression of P16 ($P = 0.033$; Fig. 3a) and P53 expression ($P = 0.008$; Fig. 3b) were found to be prognostic markers for poor overall survival of patients. In contrast, absence of PTEN ($P = 0.022$; Fig. 3c) in patient RXs was significantly associated with worse overall survival. Expression of maspin (Fig. 3d), Ki67 (Fig. 3e) and BMI1 (Fig. 3f) in RXs was not significantly associated with overall survival prognosis. Due to the fact that P16 was present in the "cytoplasm only" or in the "cytoplasm and nucleus" of some samples, we sought to see if there is a difference in survival rates between these two subgroups. However, Kaplan-Meier survival analysis did not yield a difference in survival probability according to the subcellular localization of P16 (see Additional file 2).

Cox regression analysis (Table 2A) demonstrated that no significant contribution of any biomarker was detected in BXs (data not shown), but in RXs, absence of P16 expression ($P = 0.003$; $HR = 0.067$; 95 % CI: 0.011 - 0.397) was a significant favorable prognostic factor for overall survival. The other biomarkers were not found to be associated with overall survival (Table 2). Similarly, clinicopathologic parameters such as age, gender, tumor location or tumor subtype possessed no prognostic value for patient survival in multivariate analyses.

Chemotherapy response

Chemotherapy response of the tumor following chemotherapy has a strong influence on patient survival prognosis. Therefore we used binary logistic regression to analyze the expression of biomarkers on BXs in connection with gender, patient age, location of tumor and histological subtype to determine the predictive value on tumor response. Female gender was the only significant predictive factor for good chemotherapy response after neoadjuvant chemotherapy using multivariate analysis for models with BMI1, Ki67, PTEN, P16 and P53 (data not shown). In the multivariate model established for maspin, no such link between female gender and good chemotherapy response was detected.

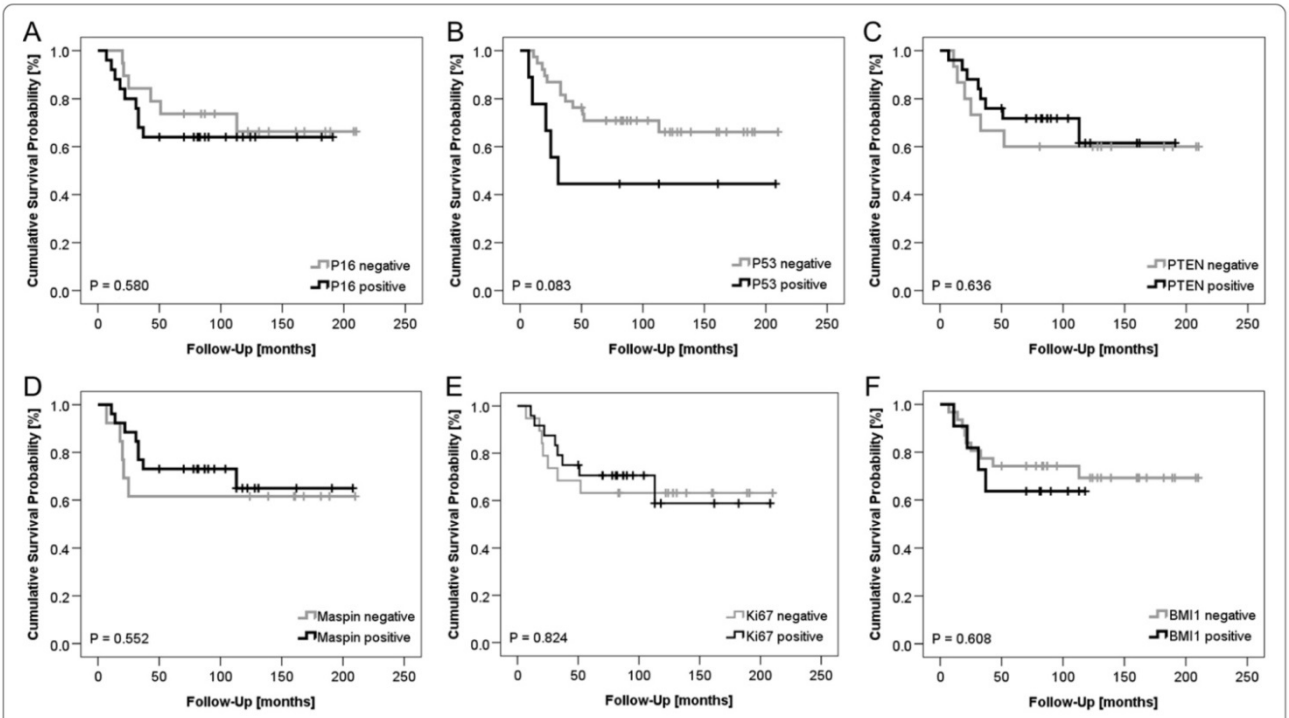


Fig. 2 Univariate Kaplan-Meier survival analysis of biomarkers in BXs. Kaplan-Meier survival curves showing survival probabilities of patients according to their (**a**) P16, (**b**) P53, (**c**) PTEN, (**d**)x maspin, (**e**) Ki67 and (**f**) BMI1 expression status

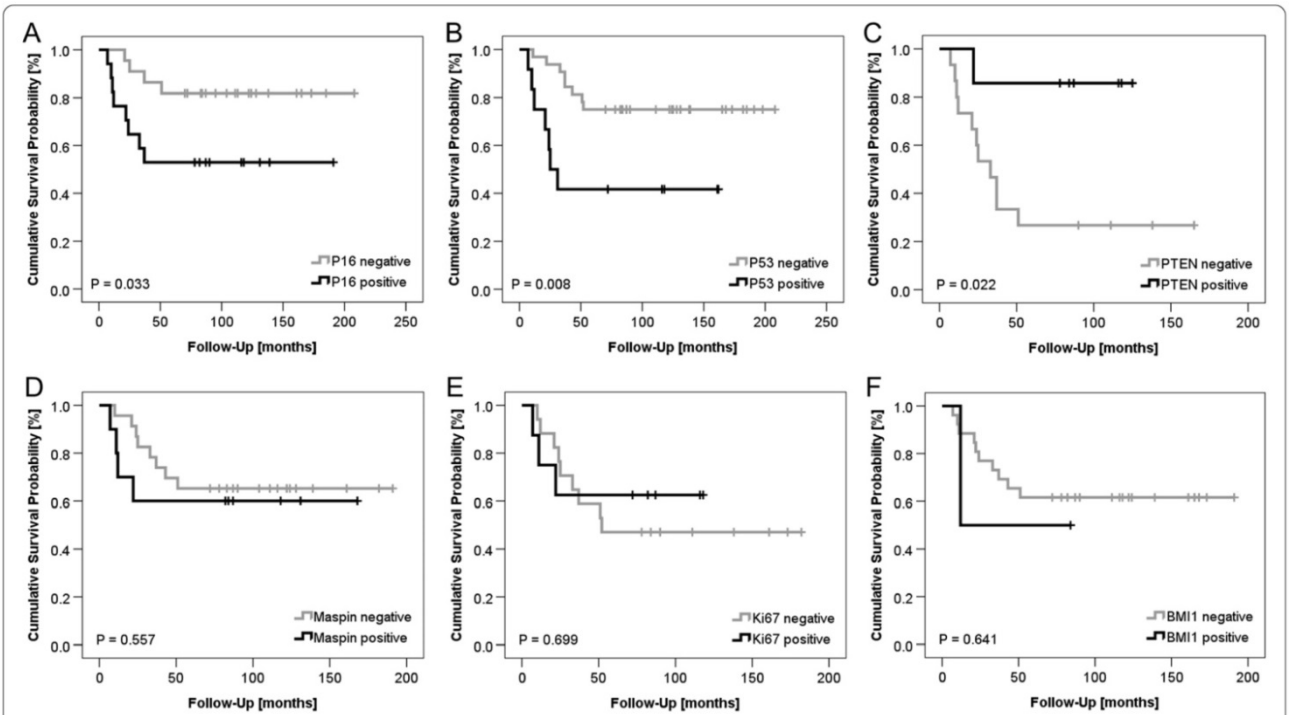


Fig. 3 Univariate Kaplan-Meier survival analysis of biomarkers in RXs. Kaplan-Meier survival curves showing survival probabilities of patients according to their (**a**) P16, (**b**) P53, (**c**) PTEN, (**d**) maspin, (**e**) Ki67 and (**f**) BMI1 – expression status

Table 2 Multivariate analysis of patients with osteosarcomas receiving neoadjuvant chemotherapy

	A. Cox regression analysis of association between clinicopathologic variables and overall survival				B. Binary logistic regression analysis of association between clinicopathologic variables and tumor-response status			
Variables[a]	P-value	HR	95 % CI		P-value	OR	95 % CI	
Age	0.973				0.126			
<10 years	0.829	0.000	0.000	∞	0.043	0.006	0.000	0.845
10–24 years	0.922	0.876	0.063	12.227	0.084	0.012	0.000	1.814
Gender	0.122	4.390	0.674	28.596	0.609	2.189	0.109	44.045
Location	0.972				0.601			
Tibia/ fibula/calcaneus	0.743	0.000	0.000	∞	0.999	6.119E9	0.000	∞
Femur	0.737	0.000	0.000	∞	0.999	4.232E9	0.000	∞
Humerus/ulna	0.719	0.000	0.000	∞	0.999	5.229E10	0.000	∞
Histological subtype	0.902				0.935			
Chondroblastic	0.986	0.139	0.000	∞	0.999	8.367E8	0.000	∞
Fibroblastic	0.922	22186	0.000	∞	0.999	0.000	0.000	∞
Osteoblastic	0.932	6308	0.000	∞	0.514	5.300	0.036	789.165
RX P16 negative ($n = 39$)	0.003	0.067	0.011	0.397	0.004	74.076	3.875	1415.946
RX P53 negative ($n = 44$)	0.315	0.515	0.141	1.879	0.159	4.786	0.541	42.368
RX PTEN negative ($n = 22$)	0.166	5.342	0.498	57.310	0.999	2.169E9	0.000	∞
RX Maspin negative ($n = 33$)	0.409	0.482	0.085	2.725	0.192	4.384	0.477	40.278
RX Ki67 negative ($n = 25$)	0.069	0.157	0.021	1.157	0.999	1.934E9	0.000	∞
RX BMI1 negative ($n = 28$)	0.353	3.624	0.239	54.951	1.000	0.000	0.000	∞

In the upper section, the multivariate analysis including P16 is shown. In the lower section, statistical parameters of biomarker expression of separate multivariate analyses (including the same clinicopathologic factors from the upper section) are shown
RX resection, *HR* hazard ratio, *OR* Odds ratio, ∞ infinity
[a]Coding of variables was a follows: age: 1 (<10 year), 2 (10–24 years) and 3 (>24 year). Gender: 1 (female) and 2 (male). Location: 1 (tibia/ fibula/ calcaneus), 2 (femur), 3 (humerus/ ulna) and 4 (axial sites). Histological subtype: 1 (chondroblastic) 2 (fibroblastic), 3 (osteoblastic) and 4 (telangiectatic)

In RXs, a correlation between tumor response after neoadjuvant chemotherapy and biomarker expression was detected. Expression of P16 in RXs was the only biomarker showing a significant correlation with poor tumor response after chemotherapy (absence of P16 in RXs: $P = 0.004$; $OR = 74.076$; 95 % CI:3.875-1415.946; Table 2B). None of the other biomarkers analyzed in RXs returned a significant correlation with tumor response.

Proliferation

In order to see if a link exists between expression of the analyzed biomarkers and proliferation, we correlated expression of P53, P16, maspin, PTEN or BMI1 with Ki67 (see Additional file 3). We found maspin expression to be positively correlating with Ki67 expression in osteosarcoma tissues ($P_{BX} < 0.001$, $P_{RX} = 0.008$; Fisher's exact test). Similarly, PTEN expression positively correlated with Ki67 expression ($P_{BX} = 0.018$, $P_{RX} = 0.046$; Fisher's exact test). Interestingly, IHC of P16 ($P_{BX} = 0.050$, $P_{RX} = 0.086$; Fisher's exact test) showed a borderline correlation whereas P53 did not significantly correlate with Ki67 on osteosarcoma samples. Despite the low number of BMI1 positive RXs, positive IHC of BMI1 on BXs showed a significantly positive correlation with the tumor proliferation marker Ki67 ($P_{BX} = 0.001$, $P_{RX} = 0.505$; Fisher's exact test).

Metastasis

Compared to primary tumor material, a higher percentage of lung metastases showed expression of the analyzed markers (examples are shown in Fig. 1, right colums). More than 50 % of positively stained samples were found in 91 % (P16), 82 % (PTEN and maspin), 60 % (Ki67) and 55 % (BMI) of all available lung metastases. In contrast, positive P53 staining was only present in 18 % of all available lung metastases.

In both BXs and RXs, no statistically significant correlations between the expression of any of the investigated biomarkers and the development of metastases during follow-up were detected. Analyses of BXs and RXs stained for P16, P53, PTEN (BXs), Ki67, Maspin and BMI1 all yielded P-values > 0.35 (Fisher's exact test) except for the analysis of PTEN in RXs, where presence of PTEN was indicative for suppression of metastases ($P = 0.063$, Fisher's exact test).

Changes of biomarkers

We investigated if changes in the histological grading of each marker (i.e., in BX and RX derived from the same

patient) had a prognostic value for patient survival. Despite a limited number of matched patient tissues available (n = 10–31, exact sample numbers indicated in Table 1), we found that changes in gradings of Ki67 (P = 0.0004, log-rank test) and maspin (P = 0.029, log-rank test) had significant prognostic value for overall survival (Fig. 4), where a decrease in grading of both markers was associated with better survival compared to no change or an increase. Changes of biomarker gradings were neither significantly correlating with the formation of metastasis nor with chemotherapy response (data not shown).

Discussion

We strongly believe that, especially in case of rare cancer entities such as osteosarcoma, all available tissue should be analyzed in order to gain more information about the molecular changes of the osteosarcoma during chemotherapy. By careful selection of the still viable resected material, valuable information can be obtained about patient survival prognosis or chemotherapy response. Here, we thus demonstrated prognostic roles of P53, P16 and PTEN in osteosarcoma by analyzing osteosarcoma samples after neoadjuvant chemotherapy. The prognostic value of P16 [53-55], P53 [15] as well as PTEN [43, 44] found in our study confirmed data found in other cancers. In addition to the common perception of analyzing chemotherapy naïve tissues in order to identify prognostic markers, we also demonstrated the value of analyzing (matching) resected tumor tissue.

To date, hardly any studies analyzing matched osteosarcoma patient samples prior to and after neoadjuvant chemotherapy exist. For the first time, we showed significant correlations between changes of maspin as well as Ki67 and osteosarcoma patient survival. In general, little is known about the role of maspin in cancer progression and it is questioned if maspin is playing a role in tumor development, in particular breast cancer, at all [48]. We are the first to study whether a metastasis-suppressing role of maspin exists in osteosarcoma, yet we could not detect a significant correlation between maspin expression in BXs or RXs and the development of metastases or any other clinicopathological parameter. However, we found that an increase in maspin expression in matched tumor specimens (prior to and after chemotherapy) had a worse survival prognosis compared to patients in which maspin gradings decreased or remained unchanged. Explanations for this finding might be either the formation of mutants of maspin [56] or the induction of maspin expression by chemotherapeutics [29] without a direct impact on tumor biology [48]. In contrast, IHC of the proliferation marker Ki67 is often discussed as a strong prognostic factor, for instance in Ewing's sarcomas [57] or breast cancer samples [38]. Despite low patient

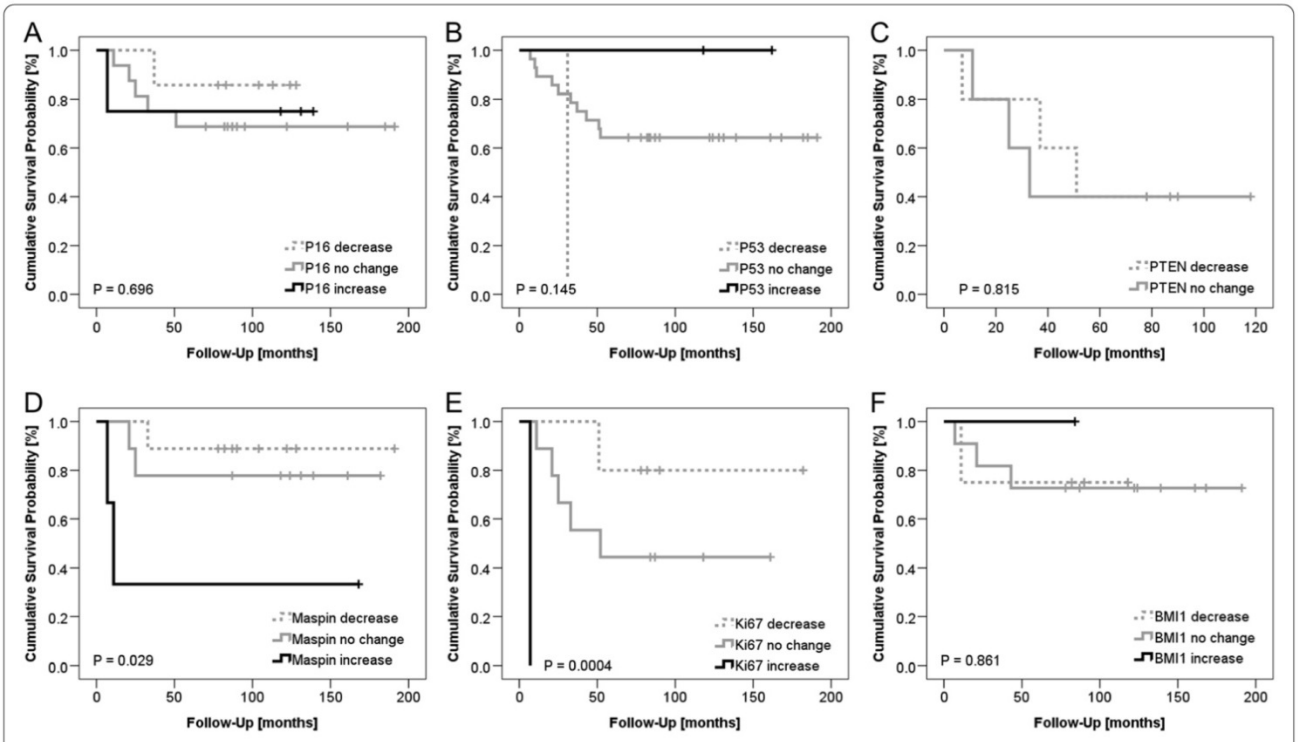


Fig. 4 Univariate Kaplan-Meier survival analysis of biomarker expression changes prior to and after neoadjuvant chemotherapy in matched tissue samples. Kaplan-Meier survival curves showing survival probabilities of patients designated by the changes of IHC gradings of (**a**) P16, (**b**) P53, (**c**) PTEN, (**d**) maspin, (**e**) Ki67 and (**f**) BMI1

numbers and although IHC of Ki67 was not significantly linked to patient survival in our and another osteosarcoma study [58], changes of Ki67 IHC scores had prognostic value for poor survival. Thus, one can speculate that osteosarcomas with increased proliferation rates might possess a survival advantage during cisplatin-based chemotherapy and ultimately lead to poorer patient survival. However, as the number of matched samples was limited, repetition of these analyses in a larger cohort should be performed.

With respect to P16, studies so far only focussed on the role of nuclear P16 in osteosarcoma. Positive expression of nuclear P16 was considered to be beneficial for overall survival rates of osteosarcoma patients [16] as well as predictive for good response after standard neoadjuvant chemotherapy [19]. These two studies investigated the role of nuclear P16 in chemotherapy naïve samples [16, 19] and showed that nuclear P16 suppressed the formation of osteosarcoma and increased the chances of success of neoadjuvant chemotherapy. Importantly, P16 in our study was predominantly present in the cytoplasm of positively stained samples, and hence, was related to a poor chemotherapy response and poor patient survival. This difference may be related by different functions of P16 in the nucleus and cytoplasm, or simply reflect the fact that cytoplasmic P16 is not available to exert its regulatory function inside the nucleus. In general, these findings are in line with studies describing other cancer types which claimed cytoplasmic P16 to be an indicator for advanced tumor stages [59] and increased aggressiveness of squamous cell carcinomas of the skin [60] or the cervix [61]. Analysis of BXs of head and neck tumors significantly linked different subcellular localizations of P16 to differences in patient survival, where strong cytoplasmic P16 was found to be prognostic for poor survival [55].

Our findings confirm findings from previous studies reporting P53 (in BXs) as a marker for osteosarcoma patient survival prognosis [15]. However, using IHC, only mutant P53 can be detected due to its prolonged half-life [62-64]. Mutant P53 is often found to be incapable of inducing DNA-damage signaling and thus, renders tumor cells apoptosis resistant. An increase in mutations of P53 might be a consequence of cisplatin-based chemotherapy [65] and proteins such as maspin might be prone to similar events and are therefore found overexpressed in osteosarcoma samples. In contrast, the P16-coding CDKN2A locus was shown to be deleted rather than mutated in osteosarcoma samples leading to a loss of P16 expression [66]. However, these results were derived from samples which were chemotherapy naïve and sequencing approaches of P16 positive RXs would be required to confirm the absence of mutations in the CDKN2A locus.

The tumor suppressor PTEN is frequently deactivated through deletions, leading to low or no PTEN expression in osteosarcoma samples [67]. Consequently, loss of PTEN leads to a more malignant phenotype and a poor patient survival. We demonstrated for the first time a better prognosis of osteosarcoma patients if their RXs were positively stained for PTEN. In line with our results, other studies also showed worse patient survival in case PTEN was absent [43, 44, 68].

Patients who present with metastatic disease have generally lower survival rates than patients with localized osteosarcomas [3]. In order to identify potential markers of metastasis, we correlated the studied biomarkers with the presence of metastases. Based on our results, none of the studied biomarkers showed any correlation with the development of metastases, irrespective of the origin of the evaluated tissue (e.g., BXs or RXs). These results point at a minor role of the studied biomarkers in the process of metastasis in osteosarcoma, although markers like P53, P16 or PTEN have a significant correlation with the overall survival of the patients included in this cohort study.

It is commonly known that males are more often affected by osteosarcoma than females [4], yet no difference in terms of response to chemotherapy has so far been shown between genders. Our analysis of osteosarcoma BXs showed a better response of female osteosarcoma-bearing patients upon neoadjuvant chemotherapy. Although the cause of this gender difference is unknown, it is unlikely to be an artefact as this outcome is in line with the generally higher survival rates of female osteosarcoma patients compared to male patients [69-71].

There are some limitations to our study. First, it is limited by low sample numbers, especially with respect to analysis of matched samples. Nevertheless, in comparison to other studies investigating biomarkers of osteosarcoma, our patient cohort can be considered as average sized [15]. Furthermore, additional analyses such as mutational analyses were not performed at this stage and although IHC is a commonly used method in the clinics it does not provide complete information about the functional state of the detected proteins. Nevertheless, IHC is a commonly available tool to identify the expression status of a protein in various tissues and to learn about the subcellular distribution of a protein within cancer cells.

Conclusions

In conclusion, using resected material, we have identified P16 and PTEN as prognostic markers for poor and for good overall survival of osteosarcoma patients, respectively. We also showed potential evidence of P16 in causing poor chemotherapy response upon neoadjuvant chemotherapy, thus establishing a basis for future research on the role of P16 in chemotherapy resistance. Importantly, the use of matched BXs and RXs also allowed us to gain more insight in the dynamics of biomarker expression following chemotherapy. Ultimately our study demonstrates

that the use of RXs yields many clues regarding chemotherapy response and patient survival, and thus, should be considered in addition to immunohistochemical evaluation of chemotherapy naïve material.

Additional files

Additional file 1: Positive, cytoplasmic only P16 immunostaining. This figure displays an immunostaining of P16 which is solely present in the cytoplasm of tumor cells (arrows point at representative cancer cells). The blue color of all nuclei is never fully covered with brown DAB reagent, showing the extranuclear localization of the P16 staining.

Additional file 2: Kaplan- Meier survival analysis of samples grouped according to subcellular localization of P16. P16 positive samples were grouped according to the subcellular localization of P16, yielding "cytoplasmic and nuclear" (cn) P16 as well as "cytoplasmic only" (c) P16. (A) Kaplan-Meier plot showing no difference in survival rates of BXs stratified according to c or cn subcellular localizations of P16 ($P = 0.358$). (B) Kaplan-Meier survival analysis using RXs yielded similar survival rates of cn and c P16 ($P = 0.845$). In contrast, cnP16 and cP16 showed worse survival rates when compared to P16 negative samples ($P = 0.067$ and $P = 0.059$, respectively). Abbreviations: BX, biopsy; c, cytoplasmic only; cn, cytoplasmic and nuclear; RX, resection.

Additional file 3: Correlations between Ki67 IHC and IHC of other biomarkers. In general, Ki67 is used as a marker for proliferation. To see if the studied putatitve tumor suppressors and BMI1 can be correlated to the proliferation state of the analyzed osteosarcoma samples, Fisher's exact tests were performed to evaluate significant correlations.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
Conception and design: BR, BF. Development of methodology: BR, CP. Grading of tumor samples: BR, CP. Analysis and interpretation of data: BR, SMB, BB, BF. Writing, review, and/or revision of the manuscript: BR, CP, SMB, BB, BF. Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): BR, BB, BF. Study supervision: BB, BF. All authors read and approved the final manuscript.

Acknowledgments
The authors thank Ivo Fuchs for creating the tissue microarray as well as the University of Zurich, the Schweizerischer Verein Balgrist (Zurich, Switzerland), the Walter L. & Johanna Wolf Foundation (Zurich, Switzerland), the Highly Specialized Medicine for Musculoskeletal Oncology program of the Canton of Zurich, the Zurcher Krebsliga (Zurich, Switzerland), the "Kind und Krebs" fund (Zollikerberg, Switzerland) and the Swiss National Science Foundation (SNF Nr.310030_149649) for financial support.

Author details
[1]Laboratory for Orthopedic Research, Department of Orthopedics, Balgrist University Hospital, Forchstrasse 340, 8008 Zurich, Switzerland. [2]Institute of Surgical Pathology, University Hospital Zurich, Zurich, Switzerland.

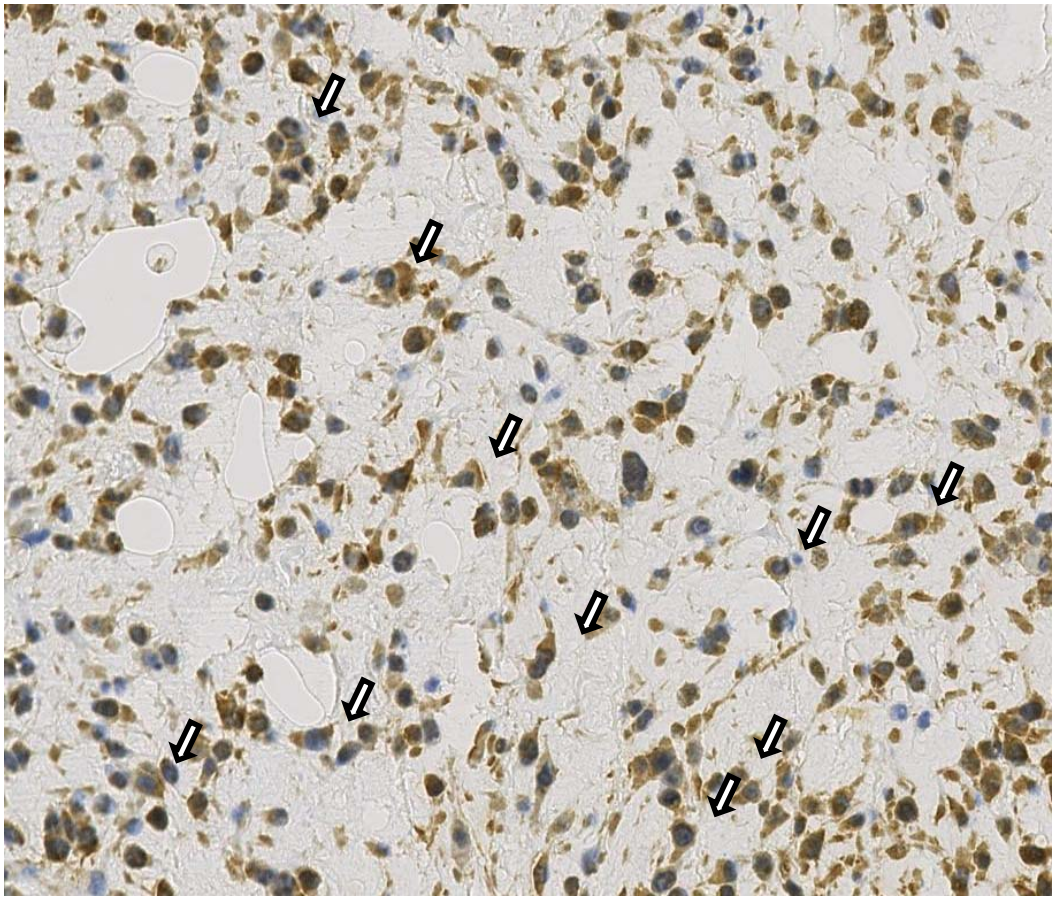
Received: 16 October 2014 Accepted: 29 April 2015
Published online: 09 May 2015

References
1. Mirabello L, Troisi RJ, Savage SA. Osteosarcoma incidence and survival rates from 1973 to 2004: data from the Surveillance, Epidemiology, and End Results Program. Cancer. 2009;115(7):1531–43.
2. Howlader N, Noone AM, Krapcho M, Garshell J, Neyman N, Altekruse SF, et al. Cancer Epidemiology in Older Adolescents and Young Adults 15 to 29 Years of Age, Including SEER Incidence and Survival: 1975–2000. In: Institute NC, editor. NIH Pub No 06–5767. Bethesda, MD: SEER Cancer Statistics Review; 2013. p. 1975–2010.
3. Allison DC, Carney SC, Ahlmann ER, Hendifar A, Chawla S, Fedenko A, et al. A meta-analysis of osteosarcoma outcomes in the modern medical era. Sarcoma. 2012;2012:704872.
4. Picci P. Osteosarcoma (osteogenic sarcoma). Orphanet J Rare Dis. 2007;2:6.
5. Goorin AM, Schwartzentruber DJ, Devidas M, Gebhardt MC, Ayala AG, Harris MB, et al. Presurgical chemotherapy compared with immediate surgery and adjuvant chemotherapy for nonmetastatic osteosarcoma: Pediatric Oncology Group Study POG-8651. J Clin Oncol. 2003;21(8):1574–80.
6. Bielack SS, Kempf-Bielack B, Delling G, Exner GU, Flege S, Helmke K, et al. Prognostic factors in high-grade osteosarcoma of the extremities or trunk: an analysis of 1,702 patients treated on neoadjuvant cooperative osteosarcoma study group protocols. J Clin Oncol. 2002;20(3):776–90.
7. Provisor AJ, Ettinger LJ, Nachman JB, Krailo MD, Makley JT, Yunis EJ, et al. Treatment of nonmetastatic osteosarcoma of the extremity with preoperative and postoperative chemotherapy: a report from the Children's Cancer Group. J Clin Oncol. 1997;15(1):76–84.
8. Hogendoorn PC, Athanasou N, Bielack S, De Alava E, Dei Tos AP, Ferrari S, et al. Bone sarcomas: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol. 2010;21 Suppl 5:v204–213.
9. Kaya M, Wada T, Akatsuka T, Kawaguchi S, Nagoya S, Shindoh M, et al. Vascular endothelial growth factor expression in untreated osteosarcoma is predictive of pulmonary metastasis and poor prognosis. Clin Cancer Res. 2000;6(2):572–7.
10. Bajpai J, Sharma M, Sreenivas V, Kumar R, Gamnagatti S, Khan SA, et al. VEGF expression as a prognostic marker in osteosarcoma. Pediatr Blood Cancer. 2009;53(6):1035–9.
11. Lugowska I, Wozniak W, Klepacka T, Michalak E, Szamotulska K. A prognostic evaluation of vascular endothelial growth factor in children and young adults with osteosarcoma. Pediatr Blood Cancer. 2011;57(1):63–8.
12. Khanna C, Wan X, Bose S, Cassaday R, Olomu O, Mendoza A, et al. The membrane-cytoskeleton linker ezrin is necessary for osteosarcoma metastasis. Nat Med. 2004;10(2):182–6.
13. Salas S, Bartoli C, Deville JL, Gaudart J, Fina F, Calisti A, et al. Ezrin and alpha-smooth muscle actin are immunohistochemical prognostic markers in conventional osteosarcomas. Virchows Arch. 2007;451(6):999–1007.
14. Li H, Min D, Zhao H, Wang Z, Qi W, Zheng S, et al. The Prognostic Role of Ezrin Immunoexpression in Osteosarcoma: A Meta-Analysis of Published Data. PLoS One. 2013;8(6), e64513.
15. Jiang L, Tao C, He A. Prognostic significance of p53 expression in malignant bone tumors: a meta-analysis. Tumour Biol. 2013;34(2):1037–43.
16. Mohseny AB, Tieken C, van der Velden PA, Szuhai K, de Andrea C, Hogendoorn PC, et al. Small deletions but not methylation underlie CDKN2A/p16 loss of expression in conventional osteosarcoma. Genes Chromosomes Cancer. 2010;49(12):1095–103.
17. Deng Z, Niu G, Cai L, Wei R, Zhao X. The prognostic significance of CD44V6, CDH11, and beta-catenin expression in patients with osteosarcoma. Biomed Res Int. 2013;2013:496193.
18. Baumhoer D, Smida J, Zillmer S, Rosemann M, Atkinson MJ, Nelson PJ, et al. Strong expression of CXCL12 is associated with a favorable outcome in osteosarcoma. Mod Pathol. 2012;25(4):522–8.
19. Borys D, Canter RJ, Hoch B, Martinez SR, Tamurian RM, Murphy B, et al. P16 expression predicts necrotic response among patients with osteosarcoma receiving neoadjuvant chemotherapy. Hum Pathol. 2012;43(11):1948–54.
20. Baumhoer D, Elsner M, Smida J, Zillmer S, Rauser S, Schoene C, et al. CRIP1 expression is correlated with a favorable outcome and less metastases in osteosarcoma patients. Oncotarget. 2011;2(12):970–5.
21. Urakawa H, Nishida Y, Naruse T, Nakashima H, Ishiguro N. Cyclooxygenase-2 overexpression predicts poor survival in patients with high-grade extremity osteosarcoma: a pilot study. Clin Orthop Relat Res. 2009;467(11):2932–8.
22. Salzer-Kuntschik M, Brand G, Delling G. Determination of the degree of morphological regression following chemotherapy in malignant bone tumors. Pathologe. 1983;4(3):135–41.
23. Rossi B, Schinzari G, Maccauro G, Scaramuzzo L, Signorelli D, Rosa MA, et al. Neoadjuvant multidrug chemotherapy including high-dose methotrexate modifies VEGF expression in osteosarcoma: an immunohistochemical analysis. BMC Musculoskelet Disord. 2010;11:34.
24. Korpi JT, Hagstrom J, Lehtonen N, Parkkinen J, Sorsa T, Salo T, et al. Expression of matrix metalloproteinases-2, −8, −13, −26, and tissue inhibitors of metalloproteinase-1 in human osteosarcoma. Surg Oncol. 2011;20(1):e18–22.
25. Wang YF, Shen JN, Xie XB, Wang J, Huang G. Expression change of ezrin as a prognostic factor in primary osteosarcoma. Med Oncol. 2011;28 Suppl 1:S636–643.

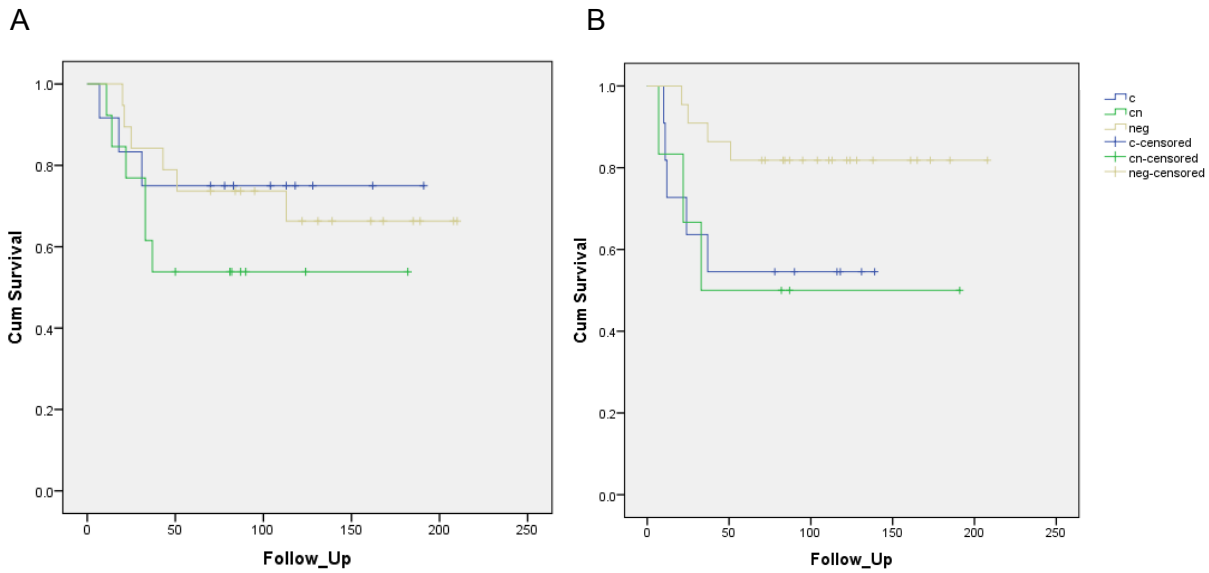
26. Bramer JA, Abudu AA, Tillman RM, Carter SR, Sumathi VP, Grimer RJ. Pre- and post-chemotherapy alkaline phosphatase levels as prognostic indicators in adults with localised osteosarcoma. Eur J Cancer. 2005;41(18):2846–52.
27. Jackson JG, Pant V, Li Q, Chang LL, Quintas-Cardama A, Garza D, et al. p53-mediated senescence impairs the apoptotic response to chemotherapy and clinical outcome in breast cancer. Cancer Cell. 2012;21(6):793–806.
28. Weller M, Rieger J, Grimmel C, Van Meir EG, De Tribolet N, Krajewski S, et al. Predicting chemoresistance in human malignant glioma cells: the role of molecular genetic analyses. Int J Cancer. 1998;79(6):640–4.
29. Ben Shachar B, Feldstein O, Hacohen D, Ginsberg D. The tumor suppressor maspin mediates E2F1-induced sensitivity of cancer cells to chemotherapy. Mol Cancer Res. 2010;8(3):363–72.
30. Oki E, Baba H, Tokunaga E, Nakamura T, Ueda N, Futatsugi M, et al. Akt phosphorylation associates with LOH of PTEN and leads to chemoresistance for gastric cancer. Int J Cancer. 2005;117(3):376–80.
31. May P, May E. Twenty years of p53 research: structural and functional aspects of the p53 protein. Oncogene. 1999;18(53):7621–36.
32. Chen X, Bahrami A, Pappo A, Easton J, Dalton J, Hedlund E, et al. Recurrent somatic structural variations contribute to tumorigenesis in pediatric osteosarcoma. Cell Rep. 2014;7(1):104–12.
33. Slade N, Moll UM. Mutational analysis of p53 in human tumors: immunocytochemistry. Methods Mol Biol. 2003;234:231–43.
34. Serrano M, Hannon GJ, Beach D. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. Nature. 1993;366(6456):704–7.
35. Mohseny AB, Szuhai K, Romeo S, Buddingh EP, Briaire-de Bruijn I, de Jong D, et al. Osteosarcoma originates from mesenchymal stem cells in consequence of aneuploidization and genomic loss of Cdkn2. J Pathol. 2009;219(3):294–305.
36. Barnard NJ, Hall PA, Lemoine NR, Kadar N. Proliferative index in breast carcinoma determined in situ by Ki67 immunostaining and its relationship to clinical and pathological variables. J Pathol. 1987;152(4):287–95.
37. Sorbye SW, Kilvaer TK, Valkov A, Donnem T, Smeland E, Al-Shibli K, et al. Prognostic Impact of Jab1, p16, p21, p62, Ki67 and Skp2 in Soft Tissue Sarcomas. PLoS One. 2012;7(10), e47068.
38. Yerushalmi R, Woods R, Ravdin PM, Hayes MM, Gelmon KA. Ki67 in breast cancer: prognostic and predictive potential. Lancet Oncol. 2010;11(2):174–83.
39. Antonarakis ES, Keizman D, Zhang Z, Gurel B, Lotan TL, Hicks JL, et al. An immunohistochemical signature comprising PTEN, MYC, and Ki67 predicts progression in prostate cancer patients receiving adjuvant docetaxel after prostatectomy. Cancer. 2012;118(24):6063–71.
40. Jacobs JJ, Kieboom K, Marino S, DePinho RA, van Lohuizen M. The oncogene and Polycomb-group gene bmi-1 regulates cell proliferation and senescence through the ink4a locus. Nature. 1999;397(6715):164–8.
41. Wu Z, Min L, Chen D, Hao D, Duan Y, Qiu G, et al. Overexpression of BMI-1 promotes cell growth and resistance to cisplatin treatment in osteosarcoma. PLoS One. 2011;6(2), e14648.
42. Weng LP, Brown JL, Eng C. PTEN coordinates G(1) arrest by down-regulating cyclin D1 via its protein phosphatase activity and up-regulating p27 via its lipid phosphatase activity in a breast cancer model. Hum Mol Genet. 2001;10(6):599–604.
43. Sano T, Lin H, Chen X, Langford LA, Koul D, Bondy ML, et al. Differential expression of MMAC/PTEN in glioblastoma multiforme: relationship to localization and prognosis. Cancer Res. 1999;59(8):1820–4.
44. Kanamori Y, Kigawa J, Itamochi H, Sultana H, Suzuki M, Ohwada M, et al. PTEN expression is associated with prognosis for patients with advanced endometrial carcinoma undergoing postoperative chemotherapy. Int J Cancer. 2002;100(6):686–9.
45. Zou Z, Anisowicz A, Hendrix MJ, Thor A, Neveu M, Sheng S, et al. Maspin, a serpin with tumor-suppressing activity in human mammary epithelial cells. Science. 1994;263(5146):526–9.
46. Lei KF, Liu BY, Jin XL, Guo Y, Ye M, Zhu ZG. Prognostic value of nuclear maspin expression for adjuvant 5-fluorouracil-based chemotherapy in advanced gastric cancer. Exp Ther Med. 2012;3(6):993–8.
47. Triulzi T, Ratti M, Tortoreto M, Ghirelli C, Aiello P, Regondi V, et al. Maspin influences response to doxorubicin by changing the tumor microenvironment organization. Int J Cancer. 2014;134(12):2789–97.
48. Teoh SS, Vieusseux J, Prakash M, Berkowicz S, Luu J, Bird CH, et al. Maspin is not required for embryonic development or tumour suppression. Nat Commun. 2014;5:3164.
49. Fletcher CDM BJ, Hogendoorn PCW, Mertens F, editors. World Health Organization Classification of Tumours of Soft Tissue and Bone. Pathology and Genetics of Tumours of Soft Tissue and Bone. 4th ed. Lyon: IARC Press; 2013.
50. Fuchs N, Bielack SS, Epler D, Bieling P, Delling G, Korholz D, et al. Long-term results of the co-operative German-Austrian-Swiss osteosarcoma study group's protocol COSS-86 of intensive multidrug chemotherapy and surgery for osteosarcoma of the limbs. Ann Oncol. 1998;9(8):893–9.
51. Bielack S, Kempf-Bielack B, Schwenzer D, Birkfellner T, Delling G, Ewerbeck V, et al. Neoadjuvant therapy for localized osteosarcoma of extremities. Results from the Cooperative osteosarcoma study group COSS of 925 patients. Klin Padiatr. 1999;211(4):260–70.
52. Bubendorf L, Nocito A, Moch H, Sauter G. Tissue microarray (TMA) technology: miniaturized pathology archives for high-throughput in situ studies. J Pathol. 2001;195(1):72–9.
53. Emig R, Magener A, Ehemann V, Meyer A, Stilgenbauer F, Volkmann M, et al. Aberrant cytoplasmic expression of the p16 protein in breast cancer is associated with accelerated tumour proliferation. Br J Cancer. 1998;78(12):1661–8.
54. Arifin MT, Hama S, Kajiwara Y, Sugiyama K, Saito T, Matsuura S, et al. Cytoplasmic, but not nuclear, p16 expression may signal poor prognosis in high-grade astrocytomas. J Neurooncol. 2006;77(3):273–7.
55. Zhao N, Ang MK, Yin XY, Patel MR, Fritchie K, Thorne L, et al. Different cellular p16(INK4a) localisation may signal different survival outcomes in head and neck cancer. Br J Cancer. 2012;107(3):482–90.
56. Jang HL, Nam E, Lee KH, Yeom S, Son HJ, Park C. Maspin polymorphism associated with apoptosis susceptibility and in vivo tumorigenesis. Int J Mol Med. 2008;22(3):333–8.
57. Lopez-Guerrero JA, Machado I, Scotlandi K, Noguera R, Pellin A, Navarro S, et al. Clinicopathological significance of cell cycle regulation markers in a large series of genetically confirmed Ewing's sarcoma family of tumors. Int J Cancer. 2011;128(5):1139–50.
58. Sorensen FB, Jensen K, Vaeth M, Hager H, Funder AM, Safwat A, et al. Immunohistochemical Estimates of Angiogenesis, Proliferative Activity, p53 Expression, and Multiple Drug Resistance Have No Prognostic Impact in Osteosarcoma: A Comparative Clinicopathological Investigation. Sarcoma. 2008;2008:874075.
59. Karim RZ, Gerega SK, Yang YH, Spillane A, Carmalt H, Scolyer RA, et al. p16 and pRb immunohistochemical expression increases with increasing tumour grade in mammary phyllodes tumours. Histopathology. 2010;56(7):868–75.
60. Nilsson K, Svensson S, Landberg G. Retinoblastoma protein function and p16INK4a expression in actinic keratosis, squamous cell carcinoma in situ and invasive squamous cell carcinoma of the skin and links between p16INK4a expression and infiltrative behavior. Mod Pathol. 2004;17(12):1464–74.
61. Acs G, Zhang PJ, McGrath CM, Acs P, McBroom J, Mohyeldin A, et al. Hypoxia-inducible erythropoietin signaling in squamous dysplasia and squamous cell carcinoma of the uterine cervix and its potential role in cervical carcinogenesis and tumor progression. Am J Pathol. 2003;162(6):1789–806.
62. Visakorpi T, Kallioniemi OP, Heikkinen A, Koivula T, Isola J. Small subgroup of aggressive, highly proliferative prostatic carcinomas defined by p53 accumulation. J Natl Cancer Inst. 1992;84(11):883–7.
63. Kerns BJ, Jordan PA, Moore MB, Humphrey PA, Berchuck A, Kohler MF, et al. p53 overexpression in formalin-fixed, paraffin-embedded tissue detected by immunohistochemistry. J Histochem Cytochem. 1992;40(7):1047–51.
64. Collier JD, Carpenter M, Burt AD, Bassendine MF. Expression of mutant p53 protein in hepatocellular carcinoma. Gut. 1994;35(1):98–100.
65. Gercel-Taylor C, Scobee JJ, Taylor DD. Effect of chemotherapy on the mutation frequency of ovarian cancer cells at the HPRT locus. Anticancer Res. 2005;25(3B):2113–7.
66. Ottaviano L, Schaefer KL, Gajewski M, Huckenbeck W, Baldus S, Rogel U, et al. Molecular characterization of commonly used cell lines for bone tumor research: a trans-European EuroBoNet effort. Genes Chromosomes Cancer. 2010;49(1):40–51.
67. Freeman SS, Allen SW, Ganti R, Wu J, Ma J, Su X, et al. Copy number gains in EGFR and copy number losses in PTEN are common events in osteosarcoma tumors. Cancer. 2008;113(6):1453–61.
68. Velickovic M, Delahunt B, McIver B, Grebe SK. Intragenic PTEN/MMAC1 loss of heterozygosity in conventional (clear-cell) renal cell carcinoma is associated with poor patient prognosis. Mod Pathol. 2002;15(5):479–85.
69. Durnali A, Alkis N, Cangur S, Yukruk FA, Inal A, Tokluoglu S, et al. Prognostic factors for teenage and adult patients with high-grade osteosarcoma: an analysis of 240 patients. Med Oncol. 2013;30(3):624.

70. Min D, Lin F, Shen Z, Zheng S, Tan L, Yu W, et al. Analysis of prognostic factors in 333 Chinese patients with high-grade osteosarcoma treated by multidisciplinary combined therapy. Asia Pac J Clin Oncol. 2013;9(1):71–9.
71. Whelan JS, Jinks RC, McTiernan A, Sydes MR, Hook JM, Trani L, et al. Survival from high-grade localised extremity osteosarcoma: combined results and prognostic factors from three European Osteosarcoma Intergroup randomised controlled trials. Ann Oncol. 2012;23(6):1607–16.

7.1.1 Additional files



Additional file 1. Positive, cytoplasmic only P16 immunostaining. This figure displays an immunostaining of P16 which is solely present in the cytoplasm of tumor cells (arrows point at representative cancer cells). The blue color of all nuclei is never fully covered with brown DAB reagent, showing the extranuclear localization of the P16 staining.



Additional file 2. Kaplan- Meier survival analysis of samples grouped according to subcellular localization of P16. P16 positive samples were grouped according to the subcellular localization of P16, yielding “cytoplasmic and nuclear” (cn) P16 as well as “cytoplasmic only” (c) P16. **(A)** Kaplan-Meier plot showing no difference in survival rates of BXs stratified according to c or cn subcellular localizations of P16 (P = 0.358). **(B)** Kaplan-Meier survival analysis using RXs yielded similar survival rates of cn and c P16 (P = 0.845). In contrast, cnP16 and cP16 showed worse survival rates when compared to P16 negative samples (P = 0.067 and P = 0.059, respectively). Abbreviations: BX, biopsy; c, cytoplasmic only; cn, cytoplasmic and nuclear; RX, resection;

Additional file 3. Correlations between Ki67 IHC and IHC of other biomarkers									
		BX				RX			
		Maspin	neg	pos		Maspin	neg	pos	
Ki67	neg		11	5	P < 0.001		12	2	P = 0.008
	pos		1	21			2	6	
		PTEN	neg	pos		PTEN	neg	pos	
Ki67	neg		10	6	P = 0.018		11	2	P = 0.046
	pos		5	19			2	4	
		P53	neg	pos		P53	neg	pos	
Ki67	neg		15	4	P = 1.000		11	6	P = 0.667
	pos		20	4			4	4	
		P16	neg	pos		P16	neg	pos	
Ki67	neg		10	7	P = 0.050		8	7	P = 0.086
	pos		6	18			1	7	
		BMI1	neg	pos		BMI1	neg	pos	
Ki67	neg		17	0	P = 0.001		11	2	P = 0.505
	pos		12	11			8	0	
Abbreviations: IHC, immunohistochemistry; neg, negative; pos, positive.									

7.1.2 Additional study: cytoplasmic p16 in osteosarcoma

Based on the relevance of cytoplasmic p16 (cytP16) in osteosarcoma therapy as demonstrated in our manuscript, we started investigating the role of cytP16 *in vitro* using human osteosarcoma cell lines. Especially the impact of cytP16 on chemoresistance was started to be investigated.

7.1.2.1 Materials and methods

7.1.2.1.1 Immunofluorescence

Cells were seeded on glass cover slips 24 h before staining, cultured in DMEM (4.5 g/l glucose)/HamF12 (1:1) medium (Invitrogen, Carlsbad, CA, USA), supplemented with 10% heat-inactivated FCS (GIBCO, Basel, Switzerland). After 10 min of fixation with 4% paraformaldehyde, cells were permeabilized with NP40 (Nonidet P40, Sigma-Aldrich, St.

Louis, MO, USA)and blocking with 3% bovine serum albumin (BSA, Sigma-Aldrich) and 0.01% Triton X-100 (Sigma-Aldrich) in PBS was performed. Anti-p16 mouse monoclonal antibody (JC8, sc-56330, Santa Cruz Biotechnology, Inc., Dallas, TX, USA; 1:100) (233), Hoechst (Invitrogen; 1:1000) and an Alexa488-coupled secondary anti-mouse antibody (Life Technologies, Carlsbad, CA, USA; 1:200) were used to visualize the localization of p16. Images were acquired using an Axio Observer Z1 microscope (Zeiss, Oberkochen, Germany) and an Andromeda spinning disc confocal microscope (FEI Munich, Gräfelfing, Germany) and processed using ImageJ v1.47 (U. S. National Institutes of Health).

7.1.2.1.2 WST-1 assay

WST-1 cell proliferation assay (Roche, Mannheim, Germany) was performed according to the manufacturer's instructions. Briefly, cells were cultured in medium for 24 h before CDDP (Sandoz) dissolved in cell culture medium was added. After 24 h and 48 h of drug treatment WST-1 reagent was added and 3 h later absorption at 450 nm using a Synergy HT spectrophotometer (BioTek, Winooski, VT, USA) was measured. Dose-response curves and IC50-values were calculated using Prism 5 v5.01 software (GraphPad Software, Inc., La Jolla, CA, USA).

7.1.2.1.3 Western blot

Cell extracts were produced using the REAP method (234) and a cell disruptor W375 sonicator (Heat Systems Ultrasonics, Inc.). Equal amounts of cells were used for extraction and extracted proteins were separated by 10% SDS-PAGE. The proteins were then transferred by semi-dry blotting to Hybond-ECL membranes (GE Healthcare, Glattbrugg, Switzerland). Actin (anti-beta actin, MAB1501, Merck Millipore, Darmstadt, Germany; 1:10 000) and p16 (JC8, Santa Cruz Biotechnology, 1:200) were detected with the indicated antibodies and visualized using horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology) as well as the Immobilon chemiluminescence substrate (Merck Millipore) and detected with a ChemiDoc Imaging System (Bio-Rad, Hercules, CA, USA).

7.1.2.2 Results

In order to characterize the protein expression of p16 in various osteosarcoma cell lines, we selected a panel of established osteosarcoma cell lines (SaOS2, G292, HuO9, MG63, 143B (HOS-derived) and U2OS) according to their p16 status (235) and evaluated p16 expression using immunofluorescence analysis (Fig. 1).

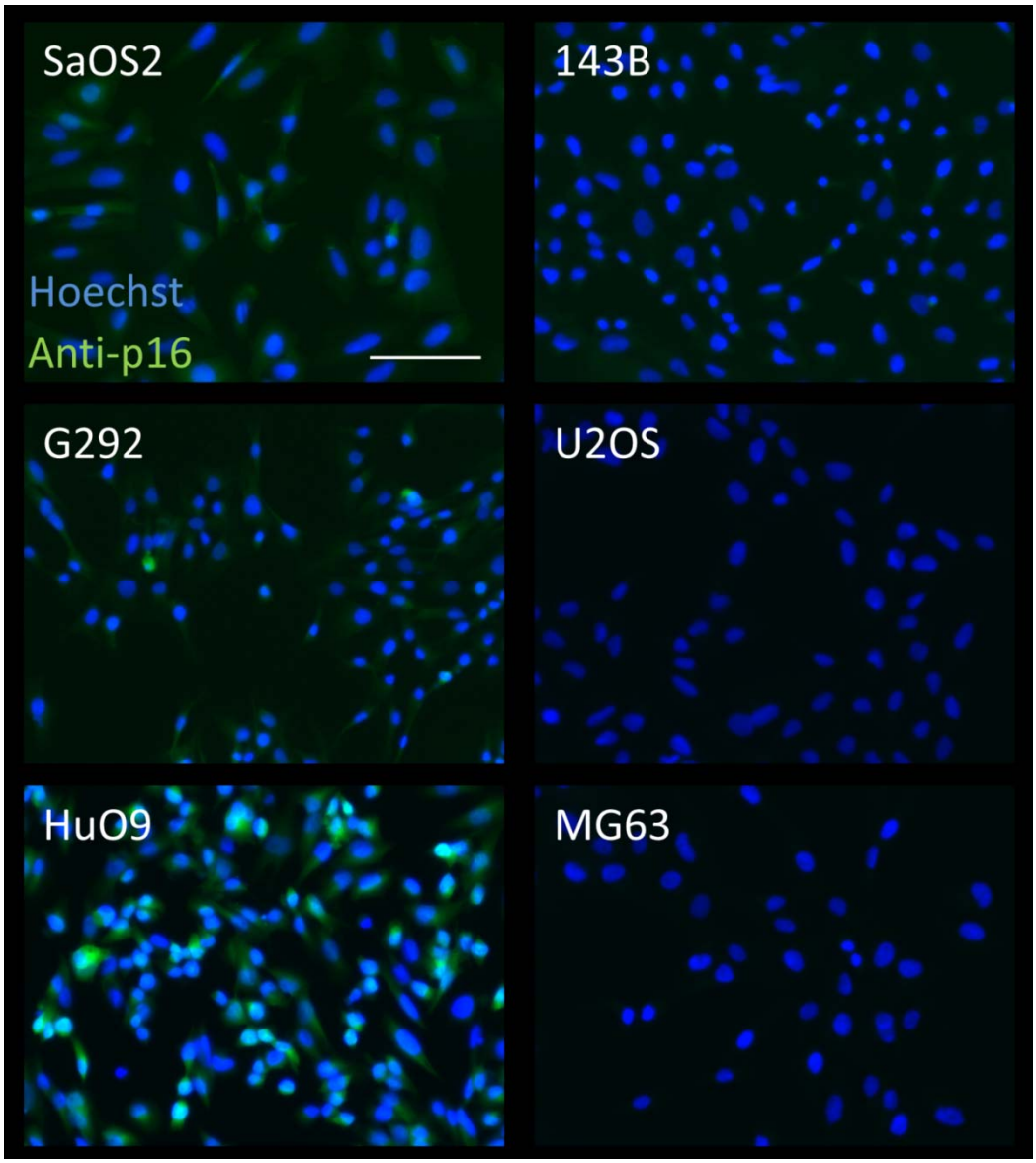


Figure 1. Immunofluorescence of six osteosarcoma cell lines. The left column shows $p16^{+}$ osteosarcoma cell lines and the right panel shows $p16^{-}$ osteosarcoma cell lines (original magnification 200 X). Scale bar corresponds to 100 µm.

After the pre-selection using fluorescence microscopy, the cytoplasmic localization of p16 in G292, HuO9 and SaOS2 was confirmed using confocal microscopy (Fig. 2).

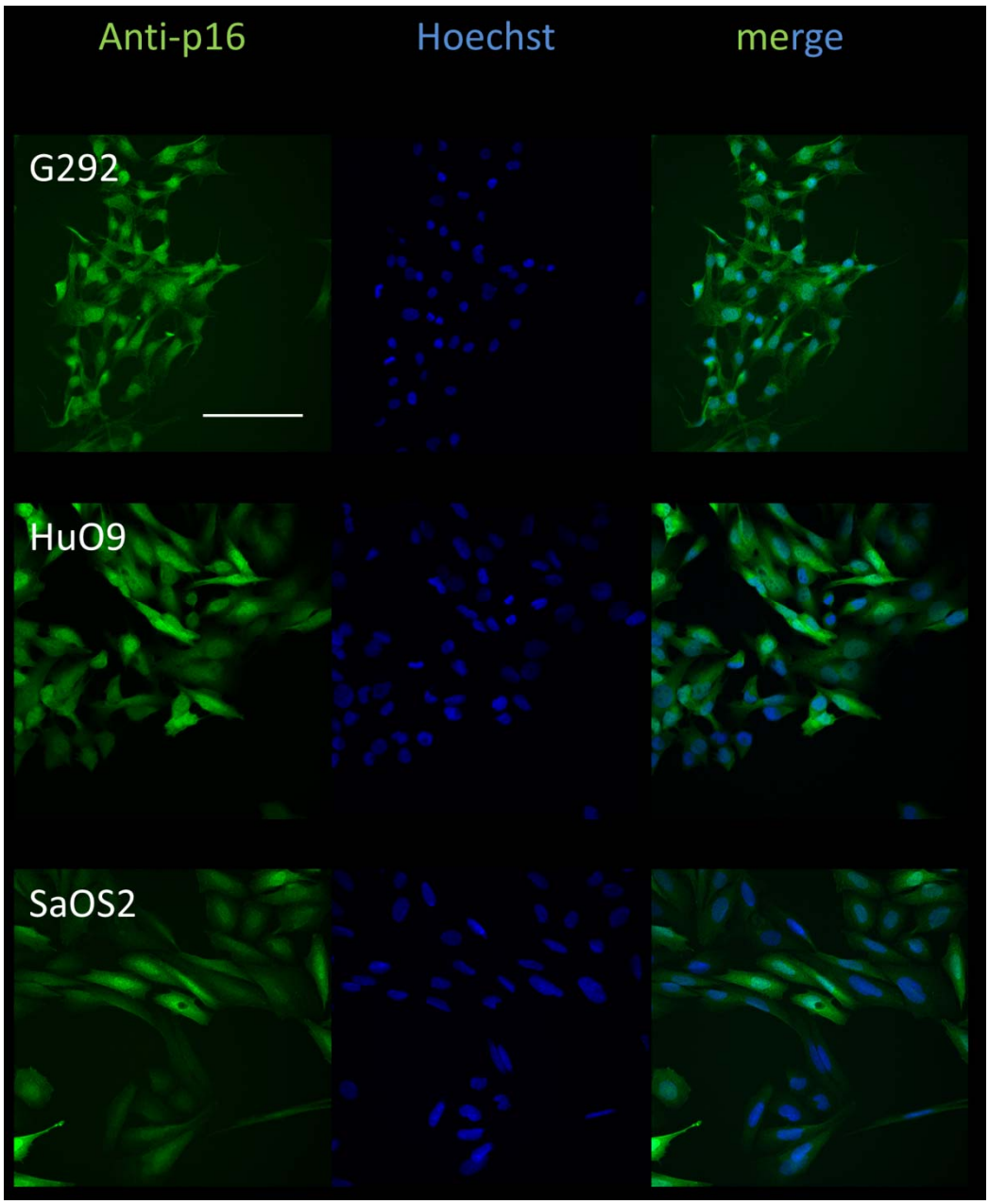


Figure 2. Immunofluorescence of cytoplasmic p16 in three osteosarcoma cell lines. Confocal microscopy images confirming the cytoplasmic subcellular localization of $p16^{+}$ cell lines (original magnification 200 X). Scale bar corresponds to 100 µm.

Next, we wanted to see if the expression of cytP16 is affected by CDDP treatment. First, the resistance of $p16^{+}$ osteosarcoma cell lines against CDDP was tested and IC_{50} values were determined using WST-1 assays (Fig. 3).

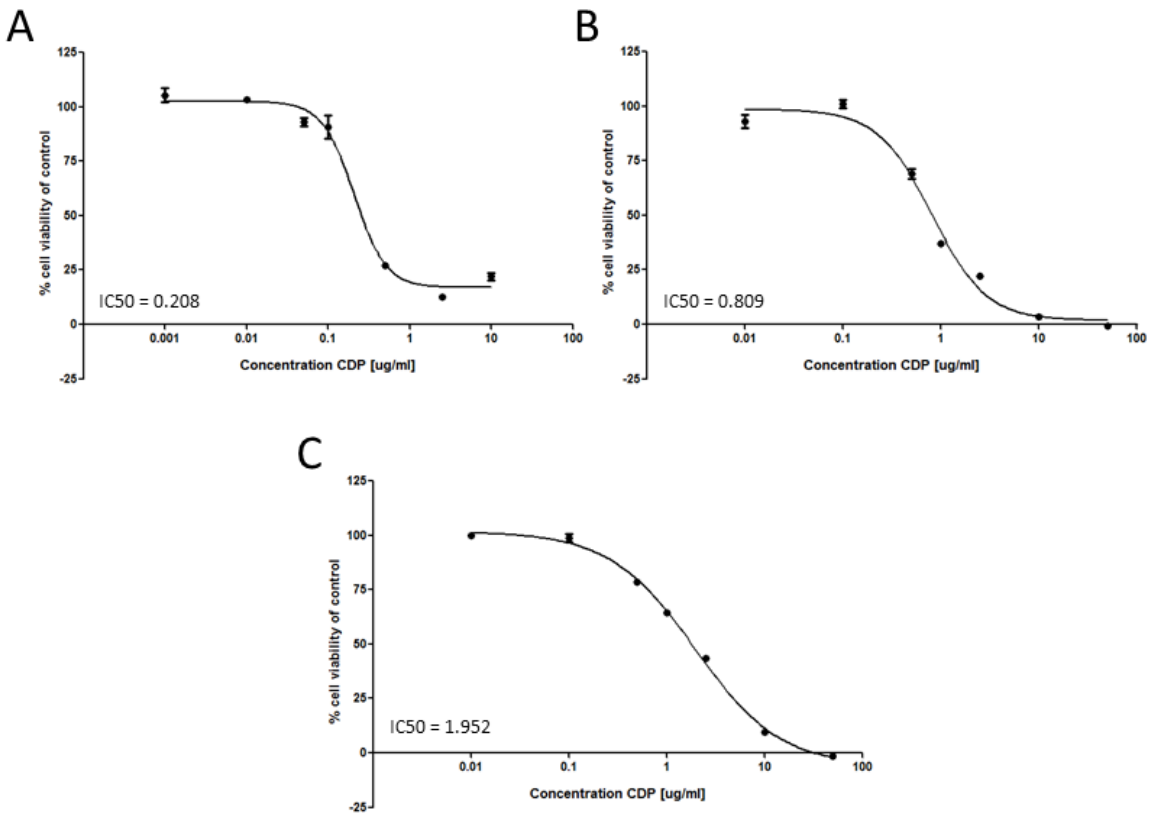


Figure 3. Dose-response curves of cytP16^{+} cell lines to CDDP treatment. Inhibition of cell growth after addition of various concentrations of CDDP was measured using WST-1 assay after 72h for A, G292; B, HuO9; and C, SaOS2.

To test whether cytP16 is affected by CDDP, we quantified the changes of protein expression after different periods of treatment with CDDP at concentrations close to the cell line-specific IC_{50} values. Therefore, western blot analysis of fractionated cell extracts was performed (Fig. 4). Quantification of the western blot analysis showed a CDDP-dependent increase of cytP16 in the cytoplasmic fraction of G292 and HuO9 cells over time compared with the corresponding control cells (Fig. 5). In contrast, SaOS2 cells did not reflect a linear increase of cytP16 over time of treatment.

Figure 4. Western blots of p16 in osteosarcoma cell lines. SaOS2 cells (black bars) were treated with two different concentrations of CDDP (0.5 µg/ml or 2 µg/ml). HuO9 (white bars) and G292 (grey bars) were treated with 0.5 µg/ml of CDDP. Whole cell extracts (WCE), cytoplasmic fractions (Cyt) and nuclear fractions (Nuc) were blotted, derived from control cells and CDDP-treated cells at different time points. Actin was used as a loading control.

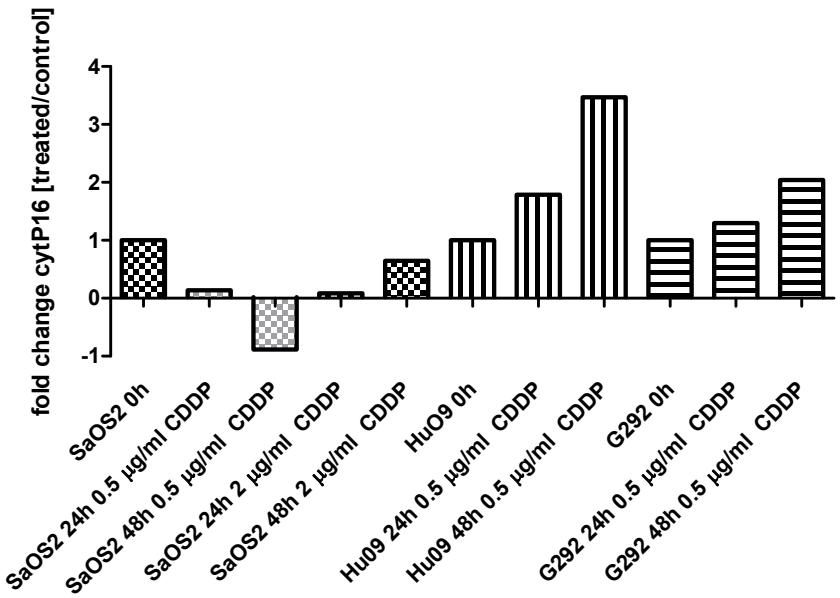


Figure 5. Quantification of western blot analysis of cytP16 after CDDP treatment. Values on the y-axis represent normalized changes of cytP16 (i.e. cytP16 / actin) with respect to the corresponding control (i.e. 24 h CDDP treated / 24 h vehicle treated). CytP16 levels of osteosarcoma cells after 0 h were normalized to 1.

7.1.3 Discussion and conclusion

The role of cytP16 in cancer is still not entirely understood. Only in the 2000s, studies demonstrated the presence of cytP16 (236), without regarding cytP16 as background-staining any longer (237). By using a specific anti-human p16 antibody (233), our results also showed that the presence of cytP16 in osteosarcoma cell lines is not an artifact. Of the few *in vitro* studies conducted on cytP16 in cancer, cytP16 was found to be present in two different forms and both were able to bind cyclin-dependent kinase 4/6 (cdk4/6) (238). It was also suggested that p16 is secreted to the cytoplasm by cancer cells to avoid its proliferation-inhibiting effects within the nucleus (239). Other studies have shown that cytP16 is capable of stimulating migration (240) or proposed to play a role in chemoresistance, yet without showing experimental evidence (241). The results from our human osteosarcoma tissue microarray (TMA) study also suggested a role of cytP16 in chemoresistance, and demonstrated a link between cytP16 and patient survival (242).

To see the influence of CDDP on cytP16, we assessed cytP16 expression levels after treatment of osteosarcoma cell lines with the corresponding IC_{50} of CDDP. Subsequently, subcellular fractionation of three cell lines possessing endogenous expression of cytP16 was performed and the amount of cytP16 was quantified. Our results showed that if Hu09 or G292 cell lines were treated with a CDDP concentration close to the IC_{50} value, the relative amounts of cytP16 increased over time, demonstrating a link between cytP16 and response to chemotherapy. In contrast, SaOS2 cells did not show a similar change of cytP16 after CDDP treatment. However, these results need to be repeated and confirmed in future studies. Additional experimental studies are required to assess the importance of altered levels of cytP16 in chemoresistance and to assess corresponding proteomic changes. Future studies might employ G292, SaOS2 and HuO9 osteosarcoma cell lines which can be induced to overexpress/ or lose cytP16 in order to study the effects of cytP16 in more detail and to decipher its precise role in chemoresistance.

7.2 Evaluation of intraarterial and intravenous cisplatin chemotherapy in the treatment of metastatic osteosarcoma using an orthotopic xenograft mouse model

Bernhard Robl[1], Sander Martijn Botter[1], Giovanni Pellegrini[2], Olga Neklyudova[1] and Bruno Fuchs[1]

[1] Laboratory for Orthopedic Research, Department of Orthopedics, Balgrist University Hospital, Zurich, Switzerland

[2] Laboratory for Animal Model Pathology, Veterinary Pathology, Vetsuisse Faculty, Zurich, Switzerland

Published in the "Journal of Experimental & Clinical Cancer Research" on the 16th of July 2016.

Citation: Robl *et al*.. J Exp Clin Cancer Res. 2016 Jul 16;35(1):113.

doi: 10.1186/s13046-016-0392-1.

Submitter's contributions to the manuscript:

Conception and design; Development of methodology; Acquisition of data; Analysis and interpretation of data; Writing of the manuscript; Administrative and material support (i.e., reporting and organizing data);

Robl et al. Journal of Experimental & Clinical Cancer Research (2016) 35:113
DOI 10.1186/s13046-016-0392-1

Journal of Experimental & Clinical Cancer Research

RESEARCH Open Access

Evaluation of intraarterial and intravenous cisplatin chemotherapy in the treatment of metastatic osteosarcoma using an orthotopic xenograft mouse model

Bernhard Robl[1], Sander Martijn Botter[1], Giovanni Pellegrini[2], Olga Neklyudova[1] and Bruno Fuchs[1*]

Abstract

Background: Osteosarcoma is the most common primary malignancy of bone. Its treatment relies on the administration of neoadjuvant and adjuvant chemotherapy combined with surgery. Alternative to common intravenous (i.v.) administration of chemotherapeutic drugs, clinical studies also evaluated the benefit of intraarterial (i.a.) administrations. However, conflicting results were obtained when both routes of administration of cisplatin (CDDP), a gold standard drug in osteosarcoma treatment, were compared. In order to overcome clinical confounding factors, we evaluated both routes of drug administration in a mouse model of experimental osteosarcoma.

Methods: We directly compared i.v. versus i.a. drug infusions of cisplatin (CDDP), in an orthotopic xenograft mouse model of metastatic osteosarcoma. We performed tumor monitoring using caliper and micro computed tomography and measured tumor perfusion using laser speckle contrast imaging. Histopathological changes were evaluated using hematoxylin and eosin staining as well as immunohistochemistry (cleaved PARP-1, CD31, HIF-1α).

Results: First, an effective concentration of 4 mg/kg i.a. CDDP was determined that significantly reduced primary tumor volume. We used this concentration of i.a. CDDP and compared it to infusions of i.v. CDDP. Systemic (i.v.) CDDP only showed minor suppression of tumor growth whereas local (i.a.) CDDP strongly inhibited tumor growth and destruction of cortical bone in the tumor-bearing hind limb. Inhibition of tumor growth was linked to a reduced blood perfusion and resulted in increased amounts of tumor necrosis after i.a. CDDP. After treatment with i.a. CDDP, remaining viable tumor tissue responded by increasing expression of HIF-1α. Side effects due to administration of CDDP were minor, showing no differences in kidney damage between i.v. and i.a. CDDP. However, increased epidermal apoptosis in the foot was an indirect marker for locally increased concentrations of CDDP.

Conclusions: Our findings demonstrate the great potential of local administration of cytotoxic chemotherapeutics, such as CDDP. Consequently, we provide a preclinical basis for a renewed interest in the clinical use of i.a. chemotherapy in osteosarcoma therapy.

Keywords: Intraarterial, Cisplatin, Osteosarcoma, Intravenous, Metastasis

* Correspondence: research@balgrist.ch
[1]Laboratory for Orthopedic Research, Department of Orthopedics, Balgrist University Hospital, Forchstrasse 340, Zurich 8008, Switzerland
Full list of author information is available at the end of the article

Background

Bone cancers are among the deadliest cancers in adolescents, with osteosarcoma as its most common representative [1, 2]. Subsequent to the introduction of chemotherapy in the early 1970s, 5-year survival rates of osteosarcoma patients with localized disease increased from 20 % to above 60 % [1, 3]. Standard of care for osteosarcoma patients currently includes systemic, intravenous (i.v.) administration of neoadjuvant chemotherapy, combined with surgical resection of the primary tumor, followed by adjuvant chemotherapy. However, 5-year survival rates plateaued at 60 % and survival rates of patients with metastatic disease remained unchanged at a low 20–30 % until today [3].

In osteosarcoma treatment, the use of neoadjuvant chemotherapy is considered valuable, yet without directly improving event-free survival compared to immediate surgery [4]. Especially pathologic analysis of the tumor response has important prognostic value. Good responders (i.e. 90 % or greater tumor necrosis) achieve up to two times better survival rates compared to patients with poor histologic responses [5–7]. In addition, neoadjuvant chemotherapy reduces tumor volumes prior to surgical resection, facilitating limb sparing procedures [8]. Therefore, neoadjuvant chemotherapy with highly cytotoxic drugs in osteosarcoma is commonly accepted as standard of care. One of the chemotherapeutics always included in today's osteosarcoma treatment regimens is cisplatin (Cis-diamminedichloroplatinum, CDDP). However, its use is limited by systemic toxicities such as ototoxicity and nephrotoxicity [9, 10].

Therefore, local, yet controlled application of CDDP may be advantageous. One way to achieve this goal is by local drug administration via the tumor feeding artery. Such intraarterial (i.a.) drug administrations were already successfully performed in the 1980s. These studies confirmed a higher bioavailability of the drug after i.a. infusion of CDDP [11–13], explaining superior histological response rates in osteosarcoma [14–16]. For instance, Wilkins *et al.* achieved a good response in 87 % of the patients if patients with localized osteosarcomas were treated with i.a. CDDP and i.v. doxorubicin [14], compared to only 41 % in similar patient cohorts where i.v. CDDP and i.v. doxorubicin were used [17], and 71 % in case of a three/four-drug regimen comprising methotrexate [6, 17–21]. In addition to better response rates, studies from the St. Luke's Medical Center achieved 10-year survival rates of between 82 and 93 % using i.a. CDDP [14, 22]. These survival rates compare favorably to other studies with maximum 10-year survival rates of, at best, 64 % with an i.v. two-drug regimen [17, 23] or up to 70 % with an i.v. three/four-drug regimen [3, 24]. Similarly, canine osteosarcoma patients showed superior responses when CDDP was infused via the tumor feeding artery compared to i.v. infusions [25].

Although these results demonstrate a clear added value of i.a. CDDP in osteosarcoma treatment, a clinical trial comparing both routes of CDDP administration was unable to show a benefit of i.a. chemotherapy [10]. This discrepancy might be explained by the design of the study, dose adaptations, administration of multiple drugs (standard of care) or its multi-institutional approach. In another study comparing i.a. versus i.v. CDDP, superior tumor responses with i.a. CDDP were only seen in the context of a three-drug regimen, and not as part of a four-drug regimen [20]. However, tumor response rates with the three-drug regimen comprising i.a. (77 %) were similar to the rates found with a four-drug regimen (81 %).

In summary, these studies demonstrate the difficulty of evaluating the "true" efficacy of i.a. CDDP due to confounding factors such as administration of different combinations of chemotherapeutics and the large difference in reported survival rates (between 50 % and 71% already for i.v. CDDP) per treatment center [6, 26]. In addition, tumor heterogeneity and side effect management make it difficult to reliably interpret the results of trials comparing both methods in a clinical setting. In this study, encouraged by the initial promising clinical benefits of i.a. chemotherapy, we investigated, under experimentally controlled conditions, the effects of local (i.a.) versus systemic (i.v.) CDDP in a preclinical mouse model of osteosarcoma.

Methods

Cell culture and transduction

Human OS 143B cells (CRL-8303) were obtained from American Type Culture Collection (ATCC, USA) and cultured in DMEM (4.5 g/L glucose)/HamF12 (1:1) medium (Invitrogen, USA) supplemented with 10 % heat-inactivated fetal calf serum at 37 °C in a humidified atmosphere containing 5 % CO_2. Previously, 143B cells were transduced with the *LacZ* gene [27]. In this study, 143B/LacZ cells were additionally infected with retroviral particles containing the mCherry sequence integrated into a pQCIXH backbone, similar as described elsewhere [28]. The original mCherry-containing pcDNA3.1 plasmid was a kind gift from Prof. M. Rudin (Institute of Biomedical Engineering, University and ETH Zurich). After transduction, 143B cells were selected in tissue culture medium with 1200 μg/ml of G418 (Merck, Germany) and 400 μg/ml of hygromycin (Merck, Germany) to stably express LacZ and mCherry.

Animal care

Female 8-week-old severe combined immunodeficiency mice (CB17/Icr-Prkdc scid/Crl; Charles River Laboratories,

Germany) were maintained in enriched individually ventilated cages with light/dark-cycles of 12 h/12 h. After delivery, animals were kept for at least a week without any interventions. Food and water was provided to the mice ad libitum. Animal care and experimental procedures were in accordance with the institutional guidelines and approved by the Ethics Committee of the Veterinary Department, Canton of Zurich, Switzerland (License Number 64/2013).

Orthotopic tumor induction in mice

143B cells were grown to subconfluence, detached with Trypsin/PBS/0.05 % EDTA, resuspended in PBS/0.05 % EDTA and kept on ice until injected. Before tumor cell injections (TCIs), mice were anesthetized using injection anesthesia. TCIs into left hind limbs were performed similar to as described elsewhere [29]. Briefly, holes were pre-drilled into the medullar cavity of left tibias using sterile needles, before 10^5 143B cells were injected. After TCIs, mice were monitored weekly for development of primary tumors (see below). Once mice started limping due to the tumor burden, 0.1 mg/kg of intraperitoneal (i.p.) Buprenorphine (Temgesic; Reckitt Benckiser, UK) was given twice daily. At the end of the study, mice were sacrificed and lung metastases were counted as described [28].

Tumor monitoring

Primary tumor monitoring

After TCIs, mice were monitored weekly using caliper and fluorescence measurements, similar as described [28]. Once human 143B osteosarcoma cells established measureable primary tumors (unambigous mCherry signal and a volume greater than 25 mm^3), drug treatment was started. mCherry tumor fluorescence was measured using an IVIS Lumina XR imaging system (Caliper Life Sciences, Inc., USA) and quantified with Living Image v3.1 software (Xenogen Corporation, USA).

Micro computed tomography

Micro computed tomography (microCT) using a SkyScan1176 microCT system (SkyScan/Bruker, Billerica, USA) equipped with a 0.5 mm aluminum filter was conducted to yield high-resolution tomographs of mouse hind limbs. Scans were obtained from each animal at the end of the study at a working source voltage of 50 kV and a source current of 500 μA yielding a final image pixel size of 17.7 μm. Frame averaging of three and exposure times of 210 ms per projection were set. Each shot required a source rotation step of 0.7° yielding scan times of approximately 8 min per mouse. Post-acquisition three-dimensional image reconstitution was done in NRecon software v1.6.9.18 (Skyscan/Bruker, USA). Reconstituted images were segmented and bone volumes were calculated using CTAn v1.13.11.0 (Skyscan/Bruker, USA). For calculation of bone and tumor volumes, the region between the distal end of the patella ("start of selection") and the bifurcation of tibia and fibula ("end of selection") was used. Bone volumes were calculated using the following formula: Δcortical bone volume = bone $volume_{tumor\text{-}limb}$ - bone $volume_{healthy\text{-}limb}$. Three-dimensional images of the mouse tibias were made in Ctvox v.2.7.0 (Skyscan/Bruker, USA).

Drug infusions

After induction anesthesia with 5 % isoflurane (Forane; AbbVie, Inc., USA), anesthesia was maintained with 2 % isoflurane during drug infusions. Mice were kept warm on a heating mat throughout the procedure. Intravenous infusions were performed via the tail vein using a 30G needle attached to a polyethylene catheter (Portex; Smiths Medical, Inc., USA) under control of a syringe pump (Legato; WPI, Inc., USA). Intraarterial infusions were performed similarly as described [30]. Briefly, after revealing the femoral artery proximal to the intratibial tumor, the femoral nerve and the femoral vein were protected by inserting a nitrile strip. Subsequently, the femoral artery was cut and in-house-made, polyethylene catheters were inserted and manually held in place. Drug (2 or 4 mg/kg CDDP; Sandoz, Austria, in 0.9 % NaCl; B. Braun Medical, Inc., Germany, containing 0.8 % patent blue V; Guerbet, France) or vehicle (0.9 % NaCl containing 0.8 % patent blue V) alone were infused in a total volume of 350 μl within 2 min under control of a syringe pump (Legato; WPI, Inc., USA) for three times (every 72 h). All manipulations were performed under a stereo microscope (SZX 10; Olympus, Inc., Japan) placed in a sterile working environment. Success of the infusion was controlled through observing the distribution of the blue dye across the hind limb. After removal of the catheter, slight pressure was applied to the injection site in order to prevent bleeding and the site of surgery was flushed with 0.9 % NaCl. The wound was closed with non-degradable silk sutures (7–0 silk; B. Braun Medical, Inc.) in an intermittent pattern. Surgical procedures for an individual i.a. drug infusion took on average 52 min.

In total, two studies were performed: 1) a "dose establishment study" to identify an effective concentration of i.a. CDDP ($N \geq 4$), and 2) a "comparison study" (4 mg/kg i.a. CDDP ($N = 11$) or i.a. vehicle ($N = 6$) versus 4 mg/kg i.v. CDDP ($N = 6$) or i.v. vehicle ($N = 6$)). Overall, i.a. infusions were tolerated well, nevertheless, one mouse treated with 2 mg/kg i.a. CDDP and two mice treated with 4 mg/kg i.a. CDDP were sacrificed prematurely during the "establishment study", due to excessive (>15 %) body weight loss. Throughout the "comparison study", one mouse of the i.v. vehicle-group had to be sacrificed due excessive body weight loss. Two mice from the group of i.v. CDDP dropped out, one during injection, another one was sacrificed due to excessive body

weight loss. One mouse of the i.a. vehicle group died during the third surgery for unknown reasons. Only one mouse from the i.a. CDDP group dropped out of the study, after being found dead in the cage for unknown reasons. Drop outs were excluded from the analysis.

Hind limb blood perfusion measurements

Laser speckle contrast imaging of the hind limbs of mice was conducted using a moorFLPI Full-Field Perfusion Imager (Moor instruments Ltd., UK) while mice were fixed in supine position. Imaging was done under low-light conditions on a heating pad set to 37 °C. Analysis of perfusion was done using the recorded flux (arbitrary units) images and the moorFLPI Review software v3.0 (Moor instruments Ltd.) by placing regions of interest (ROIs) where the primary tumor developed as well as in the corresponding region of the contralateral limb. Flux-ratios were calculated using the following formula: flux-ratio = $flux_{tumor}/flux_{contralateral}$ x 100 %.

Histological and immunohistochemical analysis

Shortly after euthanasia, primary tumors were cut in equal parts, one snap frozen and the other part decalcified, paraffin-embedded and stained using routine methods. All slides were scanned using a digital slide scanner (NAnoZoomer-XR C12000, Hamamatsu Photonics K.K., Japan) and images were obtained using the corresponding NDP.view2 software. Quantitation of tumor necrosis was conducted using frozen and paraffin-embedded, hematoxylin and eosin (H&E)-stained sections of the tumors, assessing manually the proportion of necrotic tissues versus the total amount of tumor tissue available in the sections. Immunohistochemistry (IHC) was applied on frozen tumor sections to detect apoptotic cells (anti-cleaved PARP1 rabbit monoclonal antibody, #5625S, Cell Signaling Technology, Inc., USA; 1:50), HIF-1α (anti-HIF-1α rabbit polyclonal antibody, NB100479, Novus Biologicals, LLC; USA; 1:500), CD31 (anti-PECAM-1 rabbit polyclonal antibody, sc-1506-R, Santa Cruz Biotechnology, Inc., USA; 1:1000) and Von Willebrand Factor (anti-factor VIII-related antigen (FVIII-Rag) rabbit polyclonal antibody, A0082, Dako-Agilent Technologies, Denmark; 1:100). All immunohistochemical stains were performed using a Dako Autostainer (Dako-Agilent Technologies). A minimum of five high power fields (10X magnification in NDP.view2) or if less, the maximum available tissue area were used for analysis using ImageJ v1.47 (U. S. National Institutes of Health).

In the H&E-stained kidney sections, at least 300 proximal tubules from four randomly selected cortical regions were analyzed by a veterinary pathologist (GP) and a researcher (BR) in a blinded fashion. Tubules exhibiting degenerative changes of the lining epithelial cells such as pyknosis, fragmentation and absence of the nucleus and cytoplasmic hypereosinophilia were counted and normalized to the total number of healthy tubules using ImageJ v1.47 (U. S. National Institutes of Health, USA).

Apoptotic cells in the epidermis of tumor-bearing and tumor-free hind limbs were counted by a pathologist (GP) on the digital scans of the H&E-stained sections and expressed as average number of apoptotic cells per cm of skin (2 cm of epidermis evaluated in each limb). Apoptotic keratinocytes (AKs) exhibited a small, strongly basophilic, often fragmented nucleus and a round-up intensely eosinophilic cytoplasm. Apoptosis was confirmed using IHC for cleaved caspase-3 on paraffin-embedded sections (anti-cleaved caspase 3 rabbit monoclonal antibody, #9664, Cell Signaling Technology, Inc; 1:50).

Statistical analysis

The results were given as mean ± standard error of the mean (SEM) unless otherwise stated. If Gaussian distributions were assumed, population means were compared with one-way ANOVA (for analysis of metastases, bone volume, necrosis, IHC stains) or repeated measures two-way ANOVA (for analysis of body weights, tumor volumes, blood perfusion) using Prism 5 v5.01 software (GraphPad Software, Inc., USA) followed by Bonferroni posttests. Using Prism 5, Pearson correlation calculations (HIF-1α versus CD31) as well as the Kruskal-Wallis test (tubular degeneration) and the Wilcoxon matched pairs test (number of AKs) were performed. Fisher's exact test was calculated using SPSS Statistics v22 (IBM, USA). All statistical tests were 2-sided and $p < 0.05$ was regarded as statistically significant.

Results

Establishment of an i.a. drug injection model for treating osteosarcoma

First, the concentration of i.a. CDDP that led to a significant reduction in primary tumor growth was identified, which would later on be used in comparison with i.v. CDDP. After orthotopic injection of 143B osteosarcoma cells, different concentrations of CDDP in NaCl (0.9 %) vehicle were i.a. infused into the femoral artery of the tumor-bearing limb. Only 4 mg/kg (30 ± 11 mm^3) and not 2 mg/kg (128 ± 30 mm^3) of i.a. CDDP resulted in significant retardation of tumor growth compared to the vehicle (180 ± 89 mm^3; Fig. 1a). Moreover, X-gal staining of tumor cells on the surface of lungs revealed a trend towards a dose-dependent reduction of lung metastases after i.a. CDDP (Fig. 1b). Administration of chemotherapeutics such as CDDP in preclinical models often leads to body weight loss but no significant differences between vehicle, 2 mg/kg i.a. CDDP and 4 mg/kg i.a. CDDP were noted (Fig. 1c). Every drug infusion was assessed visually by observing a color change from white to blue of the infused areas after successful infusion (Fig. 1d). Infusion

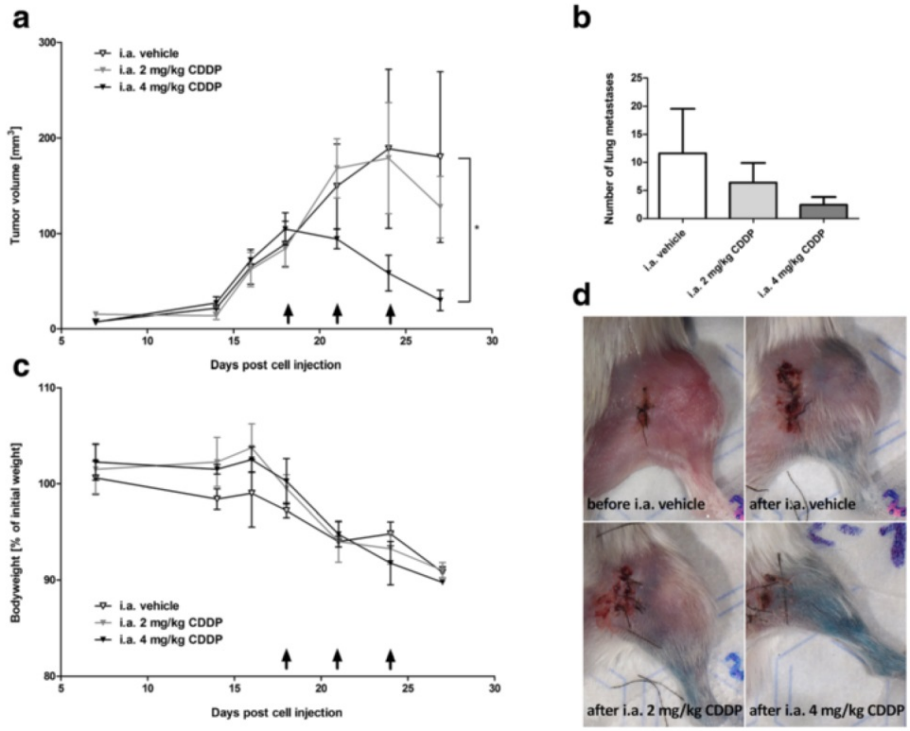


Fig. 1 Identification of an effective concentration of i.a. CDDP. **a** Tumor volumes after three separate treatments with 0, 2, or 4 mg/kg of i.a. CDDP. Tumor volumes were determined by caliper measurements. **b** Presence of pulmonary metastases after treatment with 0, 2, or 4 mg/kg of i.a. CDDP. Metastases on the surfaces of lungs were counted ex vivo after X-gal staining. **c** Changes in body weight as an indicator for general health of the mice. **d** Examples of tumor-bearing hind limbs; before the third infusion of i.a. vehicle (upper left) and after the third successful infusion of i.a. vehicle (upper right), 2 mg/kg i.a. CDDP (lower left) and 4 mg/kg CDDP (lower right). The appearance of the blue color across the leg indicated a successful infusion. Days of drug infusion are indicated by black arrows (⬆). *$p < 0.05$ as compared to the vehicle

quality controls indicated homogeneous dye distribution after three infusions of 4 mg/kg i.a. CDDP, whereas vehicle and 2 mg/kg i.a. CDDP yielded inhomogeneous dye distributions within the region of tumor growth.

Osteosarcoma development dependent on the route of CDDP administration

Next, a comparison between i.v. and i.a. CDDP infusions was conducted. Only i.a. CDDP (88 ± 31 mm^3) inhibited tumor growth and caused regression of primary tumors, while tumors continued to grow in all other treatment groups (tumor volumes at 27 days post tumor cell injection: i.v. CDDP: 307 ± 25 mm^3; i.a. vehicle: 375 ± 73 mm^3; i.v. vehicle: 491 ± 44 mm^3; two-way ANOVA: $p < 0.0001$; Fig. 2a). One day prior to sacrifice, mice were subjected to microCT scans. Tumor volumes measured within the resulting tomographs confirmed caliper measurements and yielded significantly smaller final tumor volumes in the group receiving i.a. CDDP (54 ± 35 mm^3) compared to volumes measured in other treatment groups (i.v. CDDP: 297 ± 29 mm^3; i.a. vehicle: 286 ± 58 mm^3; i.v. vehicle 479 ± 34 mm^3; ANOVA: $p < 0.0001$). Osteosarcoma is known to be associated with pathological bone remodelling and increased fracture risk, and thus, the structural integrity of the bone influences the quality of life of osteosarcoma patients. 143B cell-derived osteosarcomas were shown to behave mostly osteolytic *in vivo* and loss of cortical bone correlates with increasing tumor volume. Accordingly, administration of i.a. CDDP (87 ± 5 % of initial bone volume (before treatment)) led to the smallest loss of cortical bone compared to i.v. vehicle (75 ± 3 %), i.a. vehicle (68 ± 6 %) and i.v. CDDP (51 ± 2 %; ANOVA: $p < 0.0001$; Fig. 2b, c).

Finally, X-gal staining of lacZ tagged cells on the surface of lungs collected during necropsy (Fig. 2d) showed that systemic i.v. CDDP had no significant effect on metastatic spread towards the lung compared to i.v. vehicle control (i.v. CDDP: 202 ± 15; i.v. vehicle: 218 ± 41). In contrast, i.a. CDDP significantly reduced the number of lung metastases (i.a CDDP: 82 ± 42; i.a vehicle: 695 ± 300; ANOVA: $p < 0.001$; Fig. 2e). Of note, a nonsignificant, but on average higher amount of metastases was found in i.a. vehicle versus i.v. vehicle group.

Effect of CDDP treatment on tumor blood perfusion

Tumor-associated vasculature was assessed in vivo via blood perfusion measurements. Primary tumor growth

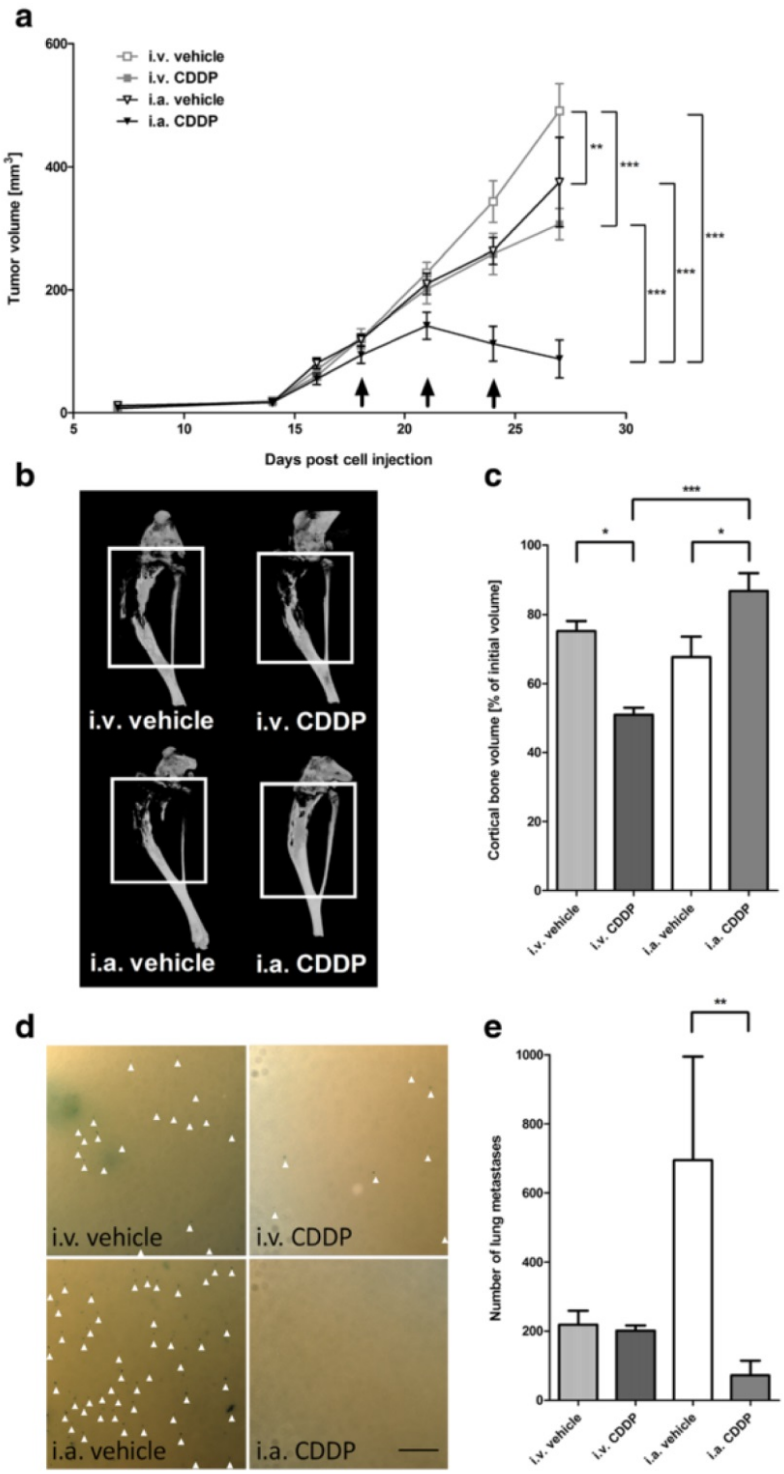


Fig. 2 Effects of different routes of CDDP administration on osteosarcoma development. **a** Tumor volumes after treatment with vehicle or 4 mg/kg CDDP, both given i.v. and i.a.. Tumor volumes were determined by caliper measurements. Days of drug infusion are indicated by black arrows (⬆). Two-way ANOVA: significant differences are only indicated for day 27. **b** Representative microCT scans of tumor-bearing bone. White squares mark the areas between the distal end of the patella and the bifurcation of tibia and fibula, which was used for quantification of differences in cortical bone. **c** Quantitation of differences in cortical (mineralized) bone volume as determined by microCT measurements. **d** Representative images of X-gal stained lung metastases. White arrowheads (Δ) indicate lacZ$^+$ lung metastases. Scale bar corresponds to 500 μm (4X). **e** Quantitation of number of pulmonary metastases on the entire lung surface. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ as compared to the indicated treatment

induced an increase in perfusion of the tumor-bearing limbs compared to the contralateral control limbs (Fig. 3a). Following i.a. CDDP, a significant decrease in perfusion compared to i.v. CDDP or i.a. vehicle was observed (two-way ANOVA: $p < 0.05$; Fig. 3b). Interestingly, the largest reduction in perfusion were detected after i.a. CDDP infusions. At the end of the study, perfusion of the i.a. CDDP-treated limbs was close to physiological values, similar to the contralateral knee region. However, areas formerly infiltrated by osteosarcomas appeared poorly perfused, indicating the occurrence of ischemic tumor necrosis (e.g. Fig. 3a: i.a. CDDP).

Histologic response to CDDP chemotherapy

Assessment of tumor necrosis after neoadjuvant chemotherapy is an established endpoint to evaluate the response to treatment in osteosarcoma patients. Figure 4a illustrates representative examples from each treatment group which were used for the analysis of tumor necrosis. In case of two animals treated with i.a. CDDP, no tumor tissue could be found on cross sections of the tumor-bearing limb, indicating a strong anti-tumor effect (100 % of tumor necrosis was assumed). The largest mean tumor necrosis was detected after i.a. CDDP (68 ± 12 %) compared with i.a. vehicle (32 ± 8 %), i.v. CDDP (17 ± 2 %) or i.v. vehicle (21 ± 3 %, ANOVA: $p < 0.01$; Fig. 4b). According to Salzer-Kuntschik, a good responder is defined by more than 90 % tumor necrosis [31]. With i.a. CDDP, a total of five (45 %) good responses was achieved, whereas no good responses were detected with i.a. vehicle, i.v. vehicle or i.v. CDDP (Fisher's exact test: $p < 0.01$; Additional file 1). Tumor cell death in the H&E-stained sections consisted of multifocal to coalescing, variably sized areas of necrosis: these areas which are, to varying extents, inherent to any rapidly growing tumor (i.e. after i.v. vehicle) are likely indicative of ischemic cell death.

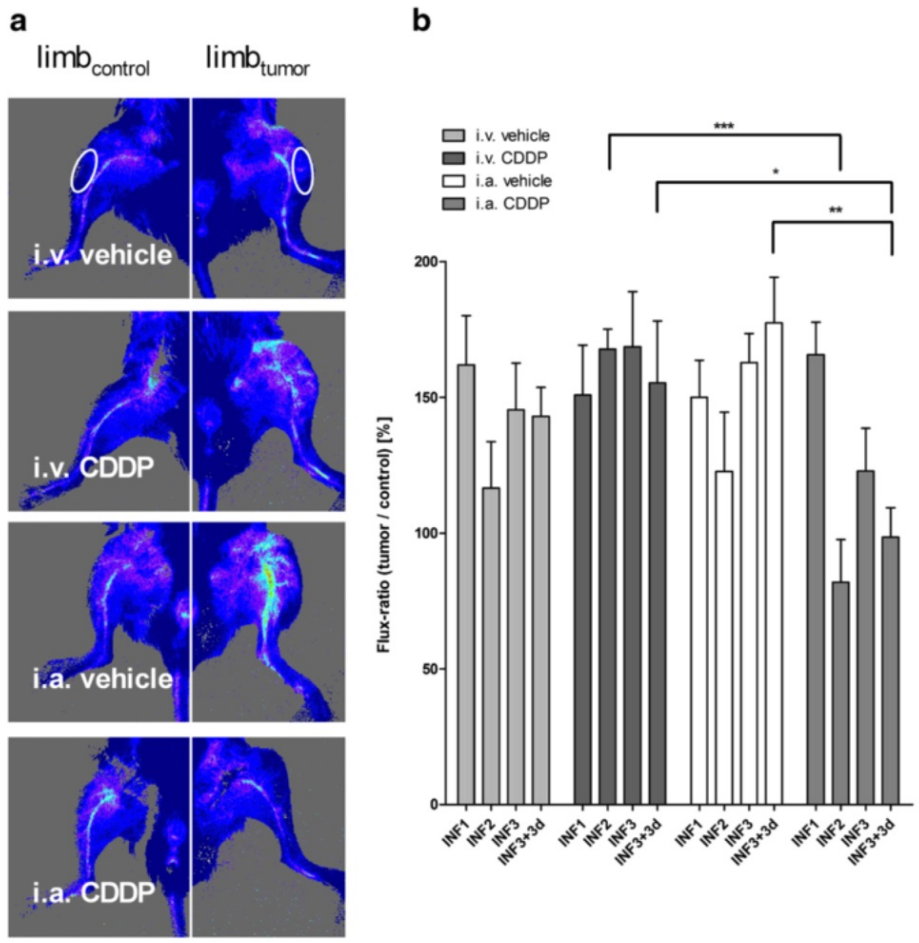


Fig. 3 Changes in hind limb blood perfusion during the treatment period. **a** Representative images of perfusion measurements of the knee region from each treatment group at the end of the study (27 days post tumor cell injection). Images in the left column illustrate healthy contralateral limbs. Perfusion images in the right column illustrate tumor-bearing limbs. Circular ROIs (only indicated for "i.v. vehicle") were used for measurements. **b** Flux-ratios of knee regions during the entire treatment period for individual treatment groups. Labeling of the x-axis indicates the respective measurement times: INF1/2/3: immediately prior to the first/s/third infusion; INF3 + 3d: three days after the final third infusion

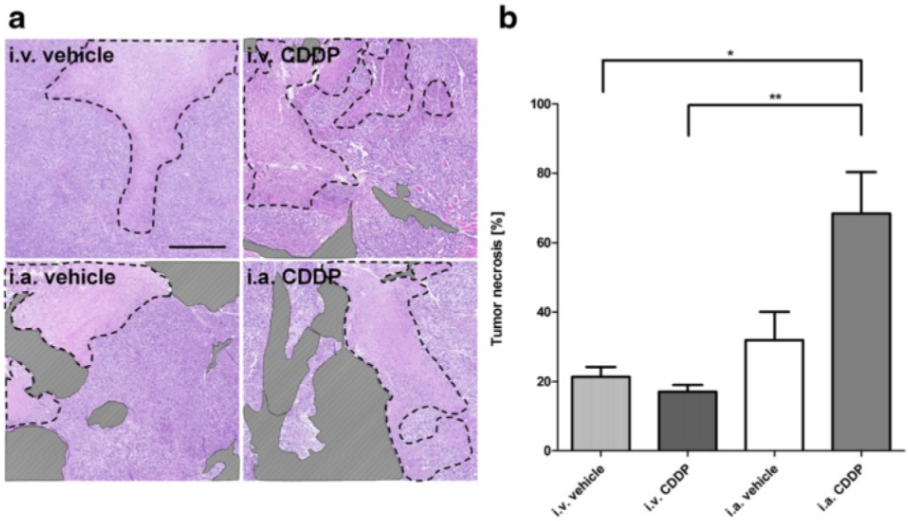


Fig. 4 Histological evaluation of tumor necrosis. **a** Representative examples of necrotic (black dashed lines) tumor areas are shown for each treatment group. Areas overlaid with a striped pattern were not considered for evaluation (e.g. bone, muscle, absence of tissue). Scale bar corresponds to 500 μm (5X). **b** Quantitation of tumor necrosis, normalized to the total tumor area which was available for analysis. *$p < 0.05$ as compared to the indicated treatment

Influence of different routes of CDDP administration on remaining viable tumor tissue

Due to the anti-tumor efficacy of i.a. CDDP, smaller areas of viable tumor were evaluated after treatment: i.a. CDDP (median 0.6 mm^2; interquartile range 0.1–4.0 mm^2) compared with i.a. vehicle (7.1 mm^2; 3.9–11.3 mm^2), i.v. CDDP (10.0 mm^2; 7.2.–12.7 mm^2) and i.v. vehicle (11.9 mm^2; 11.0–14.8 mm^2). Furthermore, two animals from the i.a. CDDP group were excluded from all immunohistochemical analyses involving viable primary tumor because of a total absence of tumor tissue. Within regions of viable tumor, scattered neoplastic cells exhibited morphological features of apoptosis, such as cell shrinkage, nuclear pyknosis and fragmentation, as indicated by immunohistochemical stains for cleaved PARP-1, a marker for chemotherapy-induced apoptosis [32]. However, no significant differences in the number of cleaved PARP-1^+ cells within areas of remaining viable tumor were detected between corresponding vehicle and treatment groups (Fig. 5a).

In order to see if the reduced limb perfusion also resulted in increased hypoxia, expression of HIF-1α, a protein expressed under sub-physiological levels of oxygen, was studied. IHC of HIF-1α demonstrated intense staining of remaining viable tumor tissue after i.a. CDDP (Fig. 5b). Furthermore, quantitation of HIF1-1α expression demonstrated a significant increase in HIF-1α levels in tumors after administration of i.a. CDDP (2.8 ± 0.7 % of remaining viable tumor tissue) compared with tumors exposed to i.a. vehicle (0.6 ± 0.6 %), i.v. CDDP (0.4 ± 0.2 %) or i.v. vehicle (0.3 ± 0.1 %; ANOVA: $p < 0.001$; Fig. 5c). High levels of HIF-1α indicate low oxygen levels, subsequently triggering neovascularization. To this end, IHC for CD31 and factor VIII-related antigen (FVIII-RAg) was performed to characterize and quantify newly formed blood vessels within the neoplasms [33]. Examples of CD31 IHC are shown in Fig. 5d. In all groups, areas of viable tumor contained negligible numbers of FVIII-Rag^+ blood vessels, while the endothelial cells lining large vessels in the skeletal muscle and connective tissue adjacent to the osteosarcomas expressed FVIII-RAg (data not shown). Quantitation of the $CD31^+$ areas within viable tumor tissue indicated a trend towards increased neovascularization after i.a. CDDP (4.1 ± 1.5 % of remaining viable tumor tissue) compared with the lower levels observed after i.a. vehicle (1.8 ± 0.7 %), i.v. CDDP (1.6 ± 0.2 %) or i.v. vehicle treatment (1.3 ± 0.2 %; Fig. 5e). Furthermore, CD31 IHC significantly correlated with HIF-1α IHC (Pearson's r: $p < 0.01$; r = 0.62; Fig. 5f).

Side effects associated with different routes of CDDP administration

Similar to the "dose establishment study", body weights were measured at regular intervals throughout the "comparison study" (Fig 6a). When comparing CDDP administrations only (i.a. CDDP: 88 ± 2 % of body weight normalized to the weight at day of tumor cell injection versus i.v. CDDP: 85 ± 1 %), no significant difference in body weight development between i.a. or i.v. CDDP administration was demonstrated. In contrast, i.v. CDDP caused a significant drop in body weight compared with i.v. vehicle (97 ± 2 %), whereas no difference was found between i.a. CDDP and i.a. vehicle (90 ± 2 %; two-way ANOVA: $p < 0.0001$; Fig. 6a).

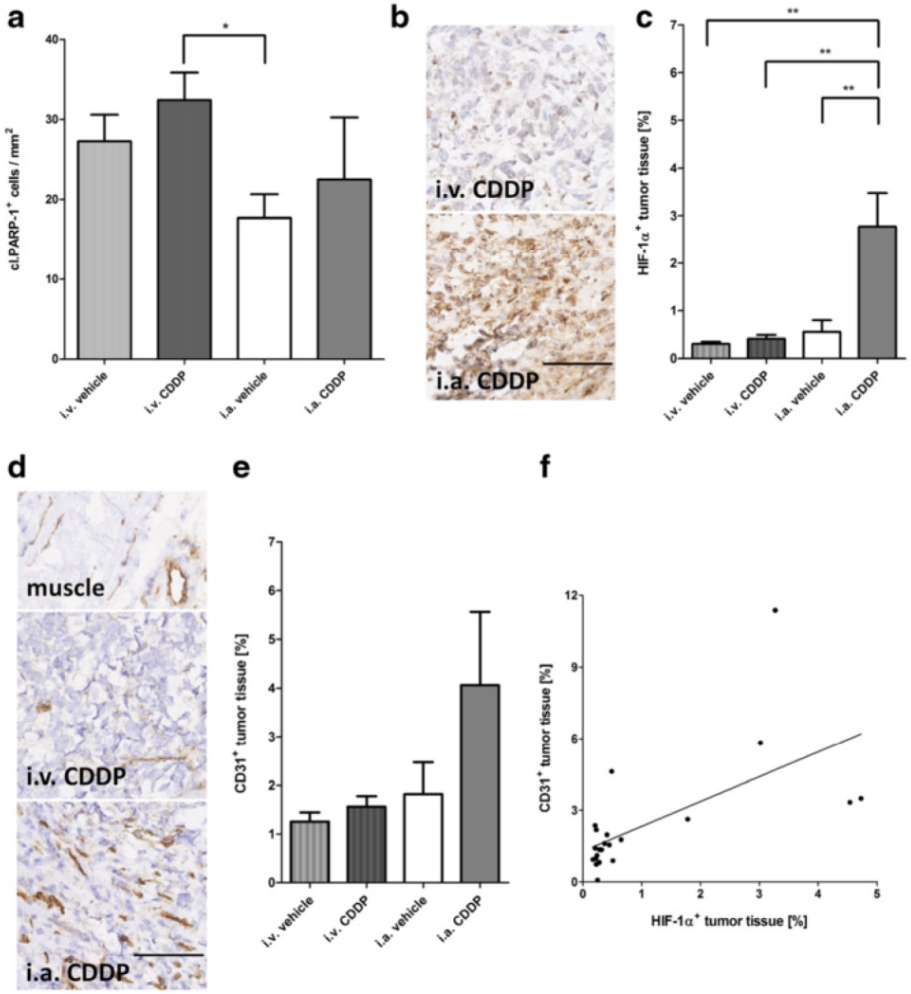


Fig. 5 Effects of different routes of CDDP administration on remaining viable tumor tissue. **a** Number of cleaved PARP-1$^+$ tumor cells. PARP-1$^+$ tumor cells were only counted within areas of viable tumor tissue. **b** Representative images of tumor tissue (i.v. CDDP, i.a. CDDP) stained for HIF-1α (20X). Scale bar corresponds to 100 μm. **c** Quantitation of HIF-1α$^+$ tumor tissue normalized to the entire viable tumor tissue available for evaluation. **d** Representative images of healthy adjacent tissue (muscle) and tumor tissue (i.v. CDDP, i.a. CDDP) stained for CD31 (20X), where the upper image shows CD31 expression in the endothelial cells lining capillaries as well as the larger vessels in the skeletal muscle surrounding the tumors, the central image represents CD31 expression after i.v. CDDP and the lower image displays an increase in CD31 expressing cells within the tumor mass after i.a. CDDP. Scale bar corresponds to 100 μm. **e** Quantitation of CD31$^+$ tumor tissue normalized to the entire tumor tissue available for evaluation. **f** Correlation of HIF-1α IHC with CD31 IHC (Pearson's r = 0.62). *$p < 0.05$; **$p < 0.01$ as compared to the indicated treatment

In general, application of CDDP is limited by a high incidence of severe nephrotoxicity characterized by degeneration or death of the proximal tubule epithelial cells [34, 35]. In this study, however, no histological abnormality was recognized in the kidneys, except in three mice after i.a. CDDP, which exhibited mild acute tubular degeneration/necrosis, affecting only a small proportion of the renal tubules (Fig. 6b).

To assess whether i.a. CDDP resulted in higher apoptotic rates of non-malignant cells, suggestive of higher local concentrations of CDDP in the operated limb, the number of apoptotic keratinocytes within the skin of treated limbs and contralateral limbs were quantified. Results demonstrated lower numbers of apoptotic keratinocytes (AK) after i.v. vehicle (0–0.05 AK/cm of skin; minimum - maximum of tumor bearing limb), i.v. CDDP (0–0.1 AK/cm) or i.a. vehicle treatment (0–0.05 AK/cm), in tumor-bearing limbs as well as in the corresponding contralateral limb (Fig. 6c). In contrast, significantly higher numbers of AKs were found after the administration of i.a. CDDP (0.35–7.6 AK/cm), yet in the tumor-bearing limb only (Wilcoxon matched pair test: $p < 0.01$; Fig. 6c).

Discussion

In this study, we present the successful establishment of i.a. drug administrations in a mouse model of experimental orthotopic osteosarcoma. Using CDDP as a gold

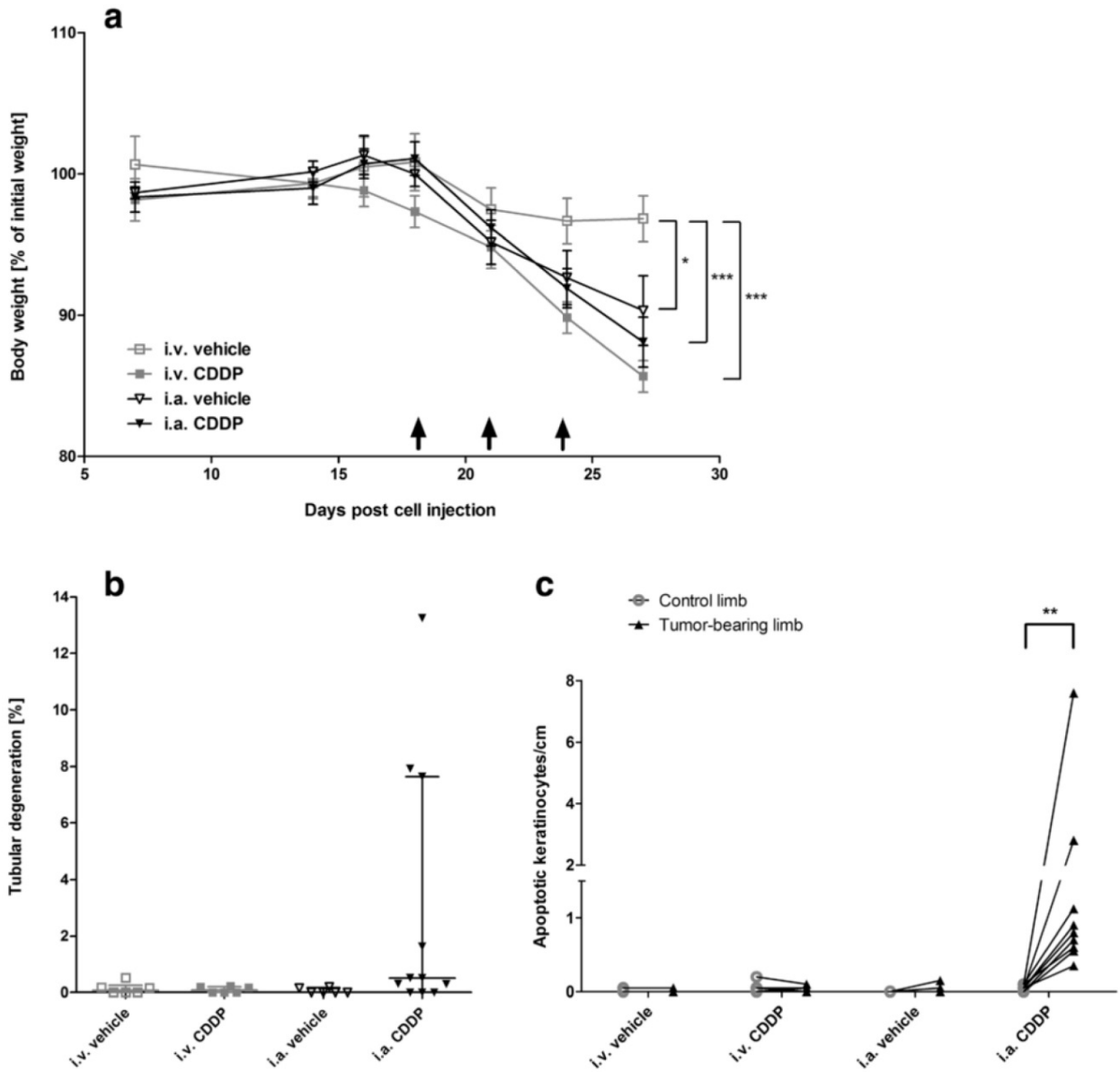


Fig. 6 Effects of CDDP treatment on the health of the mice. **a** Monitoring of body weights during the study as an indicator for general health of the mice. Days of drug/vehicle infusion are indicated by black arrows (↑). Two-way ANOVA: significant differences are only indicated for day 27. **b** Quantitation of tubular degeneration at the end of the study. Tubular degeneration is displayed as the median ± the interquartile range. **c** Apoptotic keratinocyte counts. The number of apoptotic cells per cm of epidermis of the hind limbs was determined. The number of apoptotic keratinocytes was determined in healthy limbs (control limb) as well as tumor-bearing limbs of the same animal. Data points from the same animal are connected. *$p < 0.05$; ***$p < 0.001$

standard drug for osteosarcoma treatment, we showed that our setup of i.a. drug infusions is feasible and that primary and systemic disease could be inhibited in a concentration-dependent manner. Moreover, we demonstrated that i.a. CDDP is more effective in inhibiting osteosarcoma progression than equivalent concentrations of i.v. CDDP, as indicated by smaller primary tumor volumes, decreased destruction of cortical bone as well as decreased numbers of lung metastases. Increased anti-tumor efficacy of i.a. CDDP infusions was also confirmed by histological analyses, where we demonstrated increased levels of tumor necrosis. Decreased tumor blood perfusion and increased hypoxia of the neoplasms after i.a. CDDP administration was demonstrated and explains, at least partially, the superior efficacy of localized CDDP delivery. Finally, we showed that i.a. CDDP causes increased levels of apoptotic keratinocytes in the epidermis of tumor-bearing limbs, while

other systemic side effects were similar compared with i.v. CDDP.

Despite the use of larger animal model systems such as dogs [25] or sheep [36], the most commonly used model systems in osteosarcoma research are rodents [37]. To our knowledge, this is the first report of an i.a. infusion model in experimental, orthotopic osteosarcoma in mice, where we could demonstrate superior tumor control as compared to routine i.v. infusions. Hence, our i.a. model can be used as a platform for the investigation of other small molecules whose systemic application is limited by side effects. Especially for osteosarcomas of the limb, easy access to the tumor feeding artery offers a valuable alternative to systemic CDDP infusions. However, i.a. infusions are not limited to tumors of the limb. Another recent study using a mouse model of metastatic brain tumors demonstrated advantages in tumor control with i.a. chemotherapy (internal carotid artery) compared with i.v. chemotherapy (tail vein), similar to our study [38]. This demonstrates that even difficult-to-access tumor entities can be treated with i.a. drug infusions.

Local application of small molecules such as CDDP offers several advantages compared with systemic application. First, higher tumor drug loads can be achieved after infusion of equivalent drug concentrations via the tumor-feeding artery [11], causing greater tumor necrosis [13]. In line with this assumption, we demonstrated greater tumor necrosis after i.a. CDDP compared with i.v. CDDP administration. In contrast to Winkler et al., we were able to compare i.a. versus i.v. routes of administration in the absence of clinical confounding factors such as changes in drug infusion times or stratification of patients (e.g. high-risk only) [10]. In our study, i.a. CDDP not only caused a reduction of the primary tumor volume but also minimized the loss of mineralized bone-an indicator for experimental osteosarcoma progression [39]. In contrast, loss of cortical bone was increased after i.v. CDDP treatment compared with i.v. vehicle. Likewise, when sites of bone turnover in dogs were studied, bone remodeling was significantly influenced by the systemic administration of CDDP [40].

Systemic side effects, especially nephrotoxicity, are equal or reduced after local CDDP infusion compared to systemic application without a simultaneous reduction of the systemic potency of the drug [9, 20]. In our study, most animals showed no signs of nephrotoxicity. However, we found evidence of mild nephrotoxicity, represented by scattered tubular degeneration/necrosis in three animals treated with i.a. CDDP. Renal injury was minor and unlikely to have an impact on kidney function. It is possible that hypovolemia, resulting from blood loss and/or insufficient rehydration after the repeated surgical interventions, exacerbated the observed nephrotoxicity in these animals.

Skin necrosis is another side effect observed in human patients after i.a. administration of CDDP [10, 20]. However, this usually does not lead to complications during the treatment and regenerates well. Likewise, our results indicated that i.a. CDDP led to increased numbers of apoptotic keratinocytes in the tumor-bearing limbs, but not in the healthy contralateral limbs. Elevated numbers of AK may indicate a higher local CDDP concentration in the proximity of the primary tumor and thus, help to explain the superior response after administration of i.a. CDDP compared with i.v. CDDP. Interestingly, some mild chemotherapy-induced toxicities were shown to be associated with improved osteosarcoma patient survival [23].

Consistent with the observations reported by Wilkins et al., where a reduction of the spongy tumor vasculature after i.a. CDDP was detected [14, 22], we also observed a general decrease in perfusion of the tumor region shortly after i.a. CDDP administration. This reduction in tumor perfusion in addition to the higher local CDDP concentration may have further contributed to the regression of the experimental tumors and resulted in a good histological response (as defined by having at least 90 % tumor necrosis) in at least 45 % of the mice. Thus, destruction of the tumor vasculature seems to be a necessity for i.a. CDDP to successfully induce tumor necrosis potentially resulting in a high percentage of good histologic responses [14].

The reduction in tumor perfusion after i.a. CDDP might have caused the remaining viable tumor tissue to react by expressing increased levels of HIF-1α. It is known that constitutively active HIF-1α induces neovascularization and increased expression of CD31 or VEGF [41–44]. Increased expression of HIF-1α in osteosarcomas after i.a. CDDP was paralleled by increased microvascular density assessed using IHC for CD31, a marker of immature endothelium. In addition to CD31, consecutive tissue sections were stained for FVIII-RAg, normally found in large, mature vessels [33]. The few scattered FVIII-Rag^{+} vascular structures found within the tumors were likely pre-existing and no difference was found among the different groups. In summary, our results indicate a response of the neoplasms towards ischemic damage after i.a. CDDP by increasing HIF-1α-levels and potentially initiating neovascularization.

Physical manipulation of the primary tumor as well as changes of blood perfusion within the primary tumor is known to increase numbers of circulating tumor cells and thus, the risk for the development of metastases [45, 46]. In our study, this might be reflected by a higher, albeit nonsignificant amount of lung metastases after i.a. vehicle administration, which, following i.a. CDDP, was reduced below amounts following i.v. CDDP. Thus, in addition to improved local tumor control, i.a.

CDDP also successfully controlled the number of spontaneous lung metastases. This is especially relevant in osteosarcoma, where controlling pulmonary metastases strongly influences patient survival [47–49].

One limitation of our study is the inherent variability due to any surgical procedure. Although the same surgeon performed i.a. drug infusions, the parameters of i.a. infusions varied (e.g. duration of surgery or degree of blood loss). Especially the placement of the catheter is critical for a homogeneous drug distribution in subsequent arterial branches [50] and thus, impacts success of therapy. In general, our study suggests a superior outcome in the chemotherapeutic response after i.a. delivery of CDDP, however, the individual outcomes must be interpreted alongside corresponding toxicokinetic information.

Conclusions

Taken together, our study demonstrates the potential of i.a. CDDP in a clinically relevant osteosarcoma model. The superior primary tumor control of i.a. CDDP in our study demonstrates the potential of i.a. drug administrations as currently used in some clinics. Despite the greater technical requirements for i.a. drug infusions, we suggest that the potential of i.a. infusions in osteosarcoma treatment should be considered when evaluating (novel) compounds and combinations thereof. Especially for a rare disease such as osteosarcoma, we believe that our intraarterial therapy model can aid in the preclinical assessment of drug efficacy and thus, improve osteosarcoma patient treatment.

Additional file

Additional file 1: Histological response according to treatment. Groups the histological tumor response according to current clinical criteria for evaluation of tumor necrosis. (DOCX 14 kb)

Abbreviations

AK, apoptotic keratinocyte; CD31, cluster of differentiation 31; CDDP, cisplatin; FVIII-RAg, factor VIII related antigen; H&E, hematoxylin and eosin; HIF-1α, hypoxia inducible factor-1α; i.a, intraarterial; i.p, intraperitoneal; i.v, intravenous; IHC, immunohistochemistry; microCT, micro computed tomography; PARP-1, poly ADP ribose polymerase-1; SCID, severe combined immunodeficiency; TCIs: tumor cell injections

Acknowledgments

We would like to thank Prof. Anja Kipar for fruitful discussions regarding the histological analysis of the tumor material.

Funding

Our work is supported by the University of Zurich, the Schweizerischer Verein Balgrist (Zurich, Switzerland), the Walter L. & Johanna Wolf Foundation (Zurich, Switzerland), the Highly Specialized Medicine for Musculoskeletal Oncology program of the Canton of Zurich, the Zurcher Krebsliga (Zurich, Switzerland), the "Kind und Krebs" fund (Zollikerberg, Switzerland), and the Swiss National Science Foundation SNF Nr.310030_149649.

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its additional file.

Authors' contributions

Conception and design: BR, SMB, BF. Development of methodology: BR, ON, BF. Acquisition of data: BR, GP, SMB, ON. Analysis and interpretation of data: BR, GP. Writing, review, and/or revision of the manuscript: BR, SMB, BF. Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): BR, GP, BF. Study supervision: BF. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Animal care and experimental procedures were in accordance with the institutional guidelines and approved by the Ethics Committee of the Veterinary Department, Canton of Zurich, Switzerland (License Number 64/2013).

Author details

[1]Laboratory for Orthopedic Research, Department of Orthopedics, Balgrist University Hospital, Forchstrasse 340, Zurich 8008, Switzerland. [2]Laboratory for Animal Model Pathology, Veterinary Pathology, Vetsuisse Faculty, Zurich, Switzerland.

Received: 12 May 2016 Accepted: 8 July 2016
Published online: 16 July 2016

References

1. Mirabello L, Troisi RJ, Savage SA. Osteosarcoma incidence and survival rates from 1973 to 2004: data from the Surveillance, Epidemiology, and End Results Program. Cancer. 2009;115(7):1531–43.
2. Howlader N, Noone AM, Krapcho M, Garshell J, Neyman N, Altekruse SF, Kosary CL, Yu M, Ruhl J, Tatalovich Z, Cho H, Mariotto A, Lewis DR, Chen HS, Feuer EJ, Cronin KA (eds). SEER Cancer Statistics Review, 1975-2010, Bethesda: National Cancer Institute. http://seer.cancer.gov/csr/1975_2010/, based on November 2012 SEER data submission, posted to the SEER web site, April 2013.
3. Allison DC, Carney SC, Ahlmann ER, Hendifar A, Chawla S, Fedenko A, Angeles C, Menendez LR. A meta-analysis of osteosarcoma outcomes in the modern medical era. Sarcoma. 2012;2012:704872.
4. Goorin AM, Schwartzentruber DJ, Devidas M, Gebhardt MC, Ayala AG, Harris MB, Helman LJ, Grier HE, Link MP. Presurgical chemotherapy compared with immediate surgery and adjuvant chemotherapy for nonmetastatic osteosarcoma: Pediatric Oncology Group Study POG-8651. J Clin Oncol Off J Am Soc Clin Oncol. 2003;21(8):1574–80.
5. Kager L, Zoubek A, Potschger U, Kastner U, Flege S, Kempf-Bielack B, Branscheid D, Kotz R, Salzer-Kuntschik M, Winkelmann W, et al. Primary metastatic osteosarcoma: presentation and outcome of patients treated on neoadjuvant Cooperative Osteosarcoma Study Group protocols. J Clin Oncol. 2003;21(10):2011–8.
6. Bacci G, Mercuri M, Longhi A, Ferrari S, Bertoni F, Versari M, Picci P. Grade of chemotherapy-induced necrosis as a predictor of local and systemic control in 881 patients with non-metastatic osteosarcoma of the extremities treated with neoadjuvant chemotherapy in a single institution. Eur J Cancer. 2005;41(14):2079–85.
7. Hauben EI, Weeden S, Pringle J, Van Marck EA, Hogendoorn PC. Does the histological subtype of high-grade central osteosarcoma influence the response to treatment with chemotherapy and does it affect overall survival? A study on 570 patients of two consecutive trials of the European Osteosarcoma Intergroup. Eur J Cancer. 2002;38(9):1218–25.
8. Grimer RJ. Surgical options for children with osteosarcoma. Lancet Oncol. 2005;6(2):85–92.
9. Rasch CR, Hauptmann M, Schornagel J, Wijers O, Buter J, Gregor T, Wiggenraad R, de Boer JP, Ackerstaff AH, Kroger R, et al. Intra-arterial versus

intravenous chemoradiation for advanced head and neck cancer: Results of a randomized phase 3 trial. Cancer. 2010;116(9):2159–65.
10. Winkler K, Bielack S, Delling G, Salzer-Kuntschik M, Kotz R, Greenshaw C, Jurgens H, Ritter J, Kusnierz-Glaz C, Erttmann R, et al. Effect of intraarterial versus intravenous cisplatin in addition to systemic doxorubicin, high-dose methotrexate, and ifosfamide on histologic tumor response in osteosarcoma (study COSS-86). Cancer. 1990;66(8):1703–10.
11. Stewart DJ, Benjamin RS, Zimmerman S, Caprioli RM, Wallace S, Chuang V, Calvo 3rd D, Samuels M, Bonura J, Loo TL. Clinical pharmacology of intraarterial cis-diamminedichloroplatinum(II). Cancer Res. 1983;43(2):917–20.
12. Jaffe N, Knapp J, Chuang VP, Wallace S, Ayala A, Murray J, Cangir A, Wang A, Benjamin RS. Osteosarcoma: intra-arterial treatment of the primary tumor with cis-diammine-dichloroplatinum II (CDP). Angiographic, pathologic, and pharmacologic studies. Cancer. 1983;51(3):402–7.
13. Jaffe N, Raymond AK, Ayala A, Carrasco CH, Wallace S, Robertson R, Griffiths M, Wang YM. Effect of cumulative courses of intraarterial cis-diamminedichloroplatin-II on the primary tumor in osteosarcoma. Cancer. 1989;63(1):63–7.
14. Wilkins RM, Cullen JW, Camozzi AB, Jamroz BA, Odom L. Improved survival in primary nonmetastatic pediatric osteosarcoma of the extremity. Clin Orthop Relat Res. 2005;438:128–36.
15. Hong S, Shin SJ, Jung M, Jeong J, Lee YJ, Shin KH, Roh JK, Rha SY. Comparison of long-term outcome between doublet and triplet neoadjuvant chemotherapy in non-metastatic osteosarcoma of the extremity. Oncology. 2011;80(1–2):107–17.
16. Zhou Y, Huang Z, Wu S, Zang X, Liu M, Shi J. miR-33a is up-regulated in chemoresistant osteosarcoma and promotes osteosarcoma cell resistance to cisplatin by down-regulating TWIST. J Exp Clin Cancer Res. 2014;33:12.
17. Bramwell VH, Burgers M, Sneath R, Souhami R, van Oosterom AT, Voute PA, Rouesse J, Spooner D, Craft AW, Somers R, et al. A comparison of two short intensive adjuvant chemotherapy regimens in operable osteosarcoma of limbs in children and young adults: the first study of the European Osteosarcoma Intergroup. J Clin Oncol Off J Am Soc Clin Oncol. 1992;10(10):1579–91.
18. Bielack SS, Smeland S, Whelan JS, Marina N, Jovic G, Hook JM, Krailo MD, Gebhardt M, Papai Z, Meyer J, et al. Methotrexate, Doxorubicin, and Cisplatin (MAP) Plus Maintenance Pegylated Interferon Alfa-2b Versus MAP Alone in Patients With Resectable High-Grade Osteosarcoma and Good Histologic Response to Preoperative MAP: First Results of the EURAMOS-1 Good Response Randomized Controlled Trial. J Clin Oncol Off J Am Soc Clin Oncol. 2015;33(20):2279–87.
19. Ferrari S, Smeland S, Mercuri M, Bertoni F, Longhi A, Ruggieri P, Alvegard TA, Picci P, Capanna R, Bernini G, et al. Neoadjuvant chemotherapy with high-dose Ifosfamide, high-dose methotrexate, cisplatin, and doxorubicin for patients with localized osteosarcoma of the extremity: a joint study by the Italian and Scandinavian Sarcoma Groups. J Clin Oncol Off J Am Soc Clin Oncol. 2005;23(34):8845–52.
20. Bacci G, Ferrari S, Tienghi A, Bertoni F, Mercuri M, Longhi A, Fiorentini G, Forni C, Bacchini P, Rimondini S, et al. A comparison of methods of loco-regional chemotherapy combined with systemic chemotherapy as neo-adjuvant treatment of osteosarcoma of the extremity. Eur J Surg Oncol. 2001;27(1):98–104.
21. Bielack S, Kempf-Bielack B, Schwenzer D, Birkfellner T, Delling G, Ewerbeck V, Exner GU, Fuchs N, Gobel U, Graf N, et al. Neoadjuvant therapy for localized osteosarcoma of extremities. Results from the Cooperative osteosarcoma study group COSS of 925 patients. Klin Padiatr. 1999;211(4):260–70.
22. Hugate RR, Wilkins RM, Kelly CM, Madsen W, Hinshaw I, Camozzi AB. Intraarterial chemotherapy for extremity osteosarcoma and MFH in adults. Clin Orthop Relat Res. 2008;466(6):1292–301.
23. McTiernan A, Jinks RC, Sydes MR, Uscinska B, Hook JM, van Glabbeke M, Bramwell V, Lewis IJ, Taminiau AH, Nooij MA, et al. Presence of chemotherapy-induced toxicity predicts improved survival in patients with localised extremity osteosarcoma treated with doxorubicin and cisplatin: a report from the European Osteosarcoma Intergroup. Eur J Cancer. 2012; 48(5):703–12.
24. Tunn PU, Schmidt-Peter P, Pomraenke D, Hohenberger P. Osteosarcoma in children: long-term functional analysis. Clin Orthop Relat Res. 2004;421:212–7.
25. Powers BE, Withrow SJ, Thrall DE, Straw RC, LaRue SM, Page RL, Gillette EL. Percent tumor necrosis as a predictor of treatment response in canine osteosarcoma. Cancer. 1991;67(1):126–34.
26. Petrilli AS, de Camargo B, Filho VO, Bruniera P, Brunetto AL, Jesus-Garcia R, Camargo OP, Pena W, Pericles P, Davi A, et al. Results of the Brazilian Osteosarcoma Treatment Group Studies III and IV: prognostic factors and impact on survival. J Clin Oncol off J Am Soc Clin Oncol. 2006;24(7):1161–8.
27. Gvozdenovic A, Arlt MJ, Campanile C, Brennecke P, Husmann K, Born W, Muff R, Fuchs B. Silencing of CD44 Gene Expression in Human 143-B Osteosarcoma Cells Promotes Metastasis of Intratibial Tumors in SCID Mice. PLoS One. 2013;8(4):e60329.
28. Arlt MJ, Banke IJ, Walters DK, Puskas GJ, Steinmann P, Muff R, Born W, Fuchs B. LacZ transgene expression in the subcutaneous Dunn/LM8 osteosarcoma mouse model allows for the identification of micrometastasis. J Orthop Res. 2011;29(6):938–46.
29. Husmann K, Arlt MJ, Jirkof P, Arras M, Born W, Fuchs B. Primary tumour growth in an orthotopic osteosarcoma mouse model is not influenced by analgesic treatment with buprenorphine and meloxicam. Lab Anim. 2015; 49(4):284–93.
30. Berndt K, Vogel J, Buehler C, Vogt P, Born W, Fuchs B. A new method for repeated drug infusion into the femoral artery of mice. J Am Assoc Lab Anim Sci. 2012;51(6):825–31.
31. Salzer-Kuntschik M, Brand G, Delling G. Determination of the degree of morphological regression following chemotherapy in malignant bone tumors. Pathologe. 1983;4(3):135–41.
32. Kaufmann SH, Desnoyers S, Ottaviano Y, Davidson NE, Poirier GG. Specific proteolytic cleavage of poly(ADP-ribose) polymerase: an early marker of chemotherapy-induced apoptosis. Cancer Res. 1993;53(17):3976–85.
33. Wang D, Stockard CR, Harkins L, Lott P, Salih C, Yuan K, Buchsbaum D, Hashim A, Zayzafoon M, Hardy RW, et al. Immunohistochemistry in the evaluation of neovascularization in tumor xenografts. Biotech Histochem. 2008;83(3–4):179–89.
34. Jones TW, Chopra S, Kaufman JS, Flamenbaum W, Trump BF. Cis-diamminedichloroplatinum (II)-induced acute renal failure in the rat. Correlation of structural and functional alterations. Lab Invest. 1985; 52(4):363–74.
35. Kuhlmann MK, Burkhardt G, Kohler H. Insights into potential cellular mechanisms of cisplatin nephrotoxicity and their clinical application. Nephrol Dial Transplant. 1997;12(12):2478–80.
36. Harker G, Stephens F. A report on the comparative response of sheep epidermal squamous-cell carcinoma to intraarterial versus intravenous Cisplatin infusion. Int J Oncol. 1995;7(2):365–70.
37. Guijarro MV, Ghivizzani SC, Gibbs CP. Animal models in osteosarcoma. Front Oncol. 2014;4:189.
38. Kim B, Kim K, Im KH, Kim JH, Lee JH, Jeon P, Byun H. Multiparametric MR imaging of tumor response to intraarterial chemotherapy in orthotopic xenograft models of human metastatic brain tumor. J Neurooncol. 2016; 127(2):243–51.
39. Ohba T, Cole HA, Cates JM, Slosky DA, Haro H, Ando T, Schwartz HS, Schoenecker JG. Bisphosphonates inhibit osteosarcoma-mediated osteolysis via attenuation of tumor expression of MCP-1 and RANKL. J Bone Mineral Res Off J Am Soc Bone Mineral Res. 2014;29(6):1431–45.
40. Ehrhart N, Eurell JA, Tommasini M, Constable PD, Johnson AL, Feretti A. Effect of cisplatin on bone transport osteogenesis in dogs. Am J Vet Res. 2002;63(5):703–11.
41. Elson DA, Thurston G, Huang LE, Ginzinger DG, McDonald DM, Johnson RS, Arbeit JM. Induction of hypervascularity without leakage or inflammation in transgenic mice overexpressing hypoxia-inducible factor-1alpha. Genes Dev. 2001;15(19):2520–32.
42. Mouriaux F, Sanschagrin F, Diorio C, Landreville S, Comoz F, Petit E, Bernaudin M, Rousseau AP, Bergeron D, Morcos M. Increased HIF-1alpha expression correlates with cell proliferation and vascular markers CD31 and VEGF-A in uveal melanoma. Invest Ophthalmol Vis Sci. 2014;55(3):1277–83.
43. Musumeci G, Castorina A, Magro G, Cardile V, Castorina S, Ribatti D. Enhanced expression of CD31/platelet endothelial cell adhesion molecule 1 (PECAM1) correlates with hypoxia inducible factor-1 alpha (HIF-1alpha) in human glioblastoma multiforme. Exp Cell Res. 2015;339(2):407–16.
44. Chiche J, Pommier S, Beneteau M, Mondragon L, Meynet O, Zunino B, Mouchotte A, Verhoeyen E, Guyot M, Pages G, et al. GAPDH enhances the aggressiveness and the vascularization of non-Hodgkin's B lymphomas via NF-kappaB-dependent induction of HIF-1alpha. Leukemia. 2015;29(5):1163–76.
45. Liotta LA, Kleinerman J, Saidel GM. Quantitative relationships of intravascular tumor cells, tumor vessels, and pulmonary metastases following tumor implantation. Cancer Res. 1974;34(5):997–1004.
46. Liotta LA, Saidel MG, Kleinerman J. The significance of hematogenous tumor cell clumps in the metastatic process. Cancer Res. 1976;36(3):889–94.

47. Nataraj V, Rastogi S, Khan SA, Sharma MC, Agarwala S, Vishnubhatla S, Bakhshi S. Prognosticating metastatic osteosarcoma treated with uniform chemotherapy protocol without high dose methotrexate and delayed metastasectomy: a single center experience of 102 patients. Clin Transl Oncol. 2016.
48. Rasalkar DD, Chu WC, Lee V, Paunipagar BK, Cheng FW, Li CK. Pulmonary metastases in children with osteosarcoma: characteristics and impact on patient survival. Pediatr Radiol. 2011;41(2):227–36.
49. Mizuno T, Taniguchi T, Ishikawa Y, Kawaguchi K, Fukui T, Ishiguro F, Nakamura S, Yokoi K. Pulmonary metastasectomy for osteogenic and soft tissue sarcoma: who really benefits from surgical treatment? Eur J Cardiothorac Surg. 2013;43(4):795–9.
50. van den Hoven AF, Lam MG, Jernigan S, van den Bosch MA, Buckner GD. Innovation in catheter design for intra-arterial liver cancer treatments results in favorable particle-fluid dynamics. J Exp Clin Cancer Res. 2015;34:74.

7.2.1 Additional files

Additional file 1 Histological response according to treatment

Treatment	Number	>90% tumor necrosis	50 - 90% tumor necrosis	< 50% tumor necrosis
i.a. CDDP	11	5	3	3
i.a. vehicle	6	0	2	4
i.v. CDDP	6	0	0	6
i.v. vehicle	6	0	0	6

Fisher's Exact Test: $p = 0.006$

7.3 Evaluation of F8-TNF-α in a model of early stage and progressed murine osteosarcoma

Bernhard Robl[1], Sander Martijn Botter[1], Aleksandar Boro[1], Daniela Meier[1], Dario Neri[2] and Bruno Fuchs[1,*]

1 Laboratory for Orthopedic Research, Department of Orthopedics, Balgrist University Hospital, Zurich, Switzerland

2 Institute of Pharmaceutical Sciences, ETH Zurich, Zurich, Switzerland

Submitter's contributions to the manuscript:

Conception and design; Development of methodology; Acquisition of data; Analysis and interpretation of data; Writing of the manuscript; Administrative and material support (i.e., reporting and organizing data);

See attached draft of a preliminary manuscript.

Additional work to complete the manuscript is ongoing.

Evaluation of F8-TNF-α in a model of early stage and progressed murine osteosarcoma

Short title: F8-TNF-α targeting systemic osteosarcoma

Bernhard Robl1, Sander Martijn Botter1, Aleksandar Boro1, Daniela Meier1, Dario Neri2 and Bruno Fuchs1,*

1 Laboratory for Orthopedic Research, Department of Orthopedics, Balgrist University Hospital, Zurich, Switzerland

2 Institute of Pharmaceutical Sciences, ETH Zurich, Zurich, Switzerland

BR: brobl@research.balgrist.ch

SMB: sbotter@research.balgrist.ch

AB: aboro@research.balgrist.ch

DM: dmeier@research.balgrist.ch

DN: neri@pharma.ethz.ch

BF: research@balgrist.ch

Financial support:

Our work is supported by the University of Zurich, the Schweizerischer Verein Balgrist (Zurich, Switzerland), the Walter L. & Johanna Wolf Foundation (Zurich, Switzerland), the Highly Specialized Medicine for Musculoskeletal Oncology program of the Canton of Zurich, the Zurcher Krebsliga (Zurich, Switzerland), the “Kind und Krebs” fund (Zollikerberg, Switzerland), and the Swiss National Science Foundation SNF Nr.310030_149649.

Corresponding author:

Prof. Bruno Fuchs, MD, PhD. Laboratory for Orthopedic Research, Department of Orthopedics, Balgrist University Hospital, Forchstrasse 340, 8008 Zurich, Switzerland.

Telephone: +41 (0) 44 386 16 63

Fax: +41 (0) 44 386 16 66

Email: research@balgrist.ch

Conflict of interest disclosure statement:

DN is founder and shareholder of Philogen SpA (Siena, Italy), the biotech company that owns the F8 antibody. The remaining authors declare that they have no competing financial or other conflicts of interest.

Total number of figures and tables: 6 + 1

Keywords (6): osteosarcoma, metastasis, EDA, TNF-α, amputation, immunotherapy

1 Abstract

In osteosarcoma, the most frequent cause of death is the development of pulmonary metastases. Nevertheless, most experimental drugs are evaluated by measuring primary tumor growth in various experimental systems. Extra-domain A (EDA)-targeted tumor necrosis factor α (F8-TNF) already showed promising anti-tumor effects against experimental sarcomas. Using K7M2L2, a K7M2-derived orthotopic syngeneic model of osteosarcoma, we evaluated the efficacy of F8-TNF against primary tumors and pulmonary metastases. Primary tumor growth was not affected by F8-TNF. However, numbers of pulmonary micrometastases were reduced, likely due to an F8-TNF-dependent activation of pulmonary CD4+, CD8+ and natural killer cells. Furthermore, histological analysis revealed stronger

expression of EDA in pulmonary metastases compared with primary tumor tissue. EDA expression was found to be dependent on the microenvironment as well as the growth of metastases. Immunofluorescence using human osteosarcoma tissue confirmed the presence of EDA in primary tumors as well as metastases. To further study pulmonary metastatic growth, an amputation model was established to evaluate the efficacy of F8-TNF, as single treatment or combined with doxorubicin, against established pulmonary metastases. Despite presence of EDA in metastases, no significant inhibitory effect of F8-TNF or combination treatment on further growth of late pulmonary metastases was detected and no significant difference in numbers of CD4+ or CD8+ cells in the lungs were observed. Interestingly, F8-TNF treatment led to significantly enlarged spleens, whereas addition of doxorubicin attenuated this effect. These findings demonstrate the potential of F8-TNF in reducing early metastatic growth yet suggest a lack of efficacy of F8-TNF alone or combined with doxorubicin against established pulmonary metastases. Further studies are required to improve targeted-immunotherapy against metastatic osteosarcoma.

2 Introduction

Primary bone cancers, with osteosarcoma as its most common representative, are among the deadliest cancers in children and adolescents [1, 2]. Following the introduction of chemotherapy in the 1970s, five-year survival rates of osteosarcoma increased significantly, except for patients with metastatic disease, which remained at a low 20% until today [3]. While the primary tumor can be effectively treated using modern multidrug chemotherapy schemes (i.e. doxorubicin (DOX), methotrexate, cisplatin) and surgery, metastatic disease is inefficiently treated and thus, remains to be the strongest prognostic factor for poor patient survival [4].

A promising approach against metastatic osteosarcoma may be the induction of an immune reaction towards the disseminated cancer cells. To date, several studies demonstrated

improved survival rates of osteosarcoma patients after standard chemotherapy if hallmarks of an activated immune system are present. For instance, postoperative infections in non-metastatic osteosarcoma patients [5] as well as a high ratio of intratumoral CD8+/FOX3P+ lymphocytes [6] were found to be prognostic for improved survival rates of osteosarcoma patients. In addition to improved survival rates, early recovery of lymphocytes after neoadjuvant chemotherapy was also indicative for fewer relapses [7]. Similarly, a higher pre-surgical lymphocytes to monocytes ratio was associated with better survival rates as well as a lower frequency of metastases at diagnosis [8]. Furthermore, already established chemotherapeutics such as anthracyclines (e.g. DOX) act as inducers of immunogenic cell death, further potentiating an anti-cancer immune response [9]. These studies point to a survival benefit for osteosarcoma patients mediated through the cytotoxic actions of chemotherapy on the one hand and a competent immune system on the other.

In order to further enhance chemotherapy-induced immune responses a potent cytokine can be employed. One of the most potent pro-inflammatory cytokines is tumor necrosis factor-α (TNF-α), which contributed to great anti-tumor effects against sarcomas and lymphomas already around 1900 [10, 11]. The anti-tumor effects of TNF-α depend on the tumor-bearing host [12], especially its immune activating properties [13, 14] or destruction of the tumor vasculature [14, 15]. However, systemic use of TNF-α is limited through severe cytotoxic side effects leading to shock, tissue injury and even death, already observed at TNF-α levels which can endogenously be produced by the host [16]. To overcome limiting toxicities and locally enhancing therapeutic effects, multiple moieties (e.g. NGR, F8, L19, TCP-1) were already coupled to TNF-α, with the aim of guiding it to the specific domains present in the tumor vasculature [17-20]. For instance, TNF-α linked to the F8-moiety (F8-TNF) which targets the extra-domain A (EDA) of fibronectin was already demonstrated to have strong therapeutic efficacy against established subcutaneous (s.c.) soft tissue sarcomas [18].

Another means of increasing local drug concentrations to limit systemic side effects are intraarterial (i.a.) infusions. As already demonstrated in osteosarcoma patients, intratumoral

cisplatin concentrations are elevated if the drug was infused intraarterially compared to intravenous (i.v.) administrations [21]. In addition, we also demonstrated superior tumor control using an orthotopic osteosarcoma model when i.a. cisplatin was compared with i.v. cisplatin [22]. However, not only the efficacy of cisplatin but also the efficacy of other drugs might be increased through local, i.a. administration.

In this study we investigated the potential of F8-TNF against osteosarcoma. Using a clinically relevant syngeneic K7M2L2 orthotopic mouse model of osteosarcoma, drug efficacy was first evaluated in the presence of a primary tumor and in dependence of its route of administration (i.e. i.a. versus i.v.). Next, drug efficacy against progressed osteosarcoma was assessed in an amputation model of osteosarcoma. Especially the effects of F8-TNF (alone and combined with DOX) on the circulating tumor cells (CTCs), early and late pulmonary metastases as well as on cells of the immune system were investigated.

3 Methods

3.1 Generation of the K7M2L2 cell line

Murine K7M2 osteosarcoma cells (CRL-2836) were kindly provided by Dr. Chand Khanna (Center for Cancer Research National Institute, Bethesda, MD, USA) and cultured in DMEM (4.5 g/l glucose)/HamF12 (1:1) medium (Invitrogen, Carlsbad, CA), supplemented with 10% heat-inactivated FCS (GIBCO, Basel, Switzerland), at 37°C in a humidified atmosphere containing 5% CO2. K7M2 cells were transduced with a lacZ reporter gene under control of a neomycin selection marker as described [23]. Ten µl of PBS/0.05% EDTA containing 1 x 105 of K7M2/lacZ cells were injected into the left tibia (i.t.) of female, 8-10 week old BALB/c mice as described before [23]. The K7M2L2/lacZ cell line was obtained after two rounds of in vivo selection of pulmonary metastases-derived K7M2 cells according to Fidler's method [24]. Briefly, around three weeks after intratibial (i.t.) injection of K7M2/lacZ cells, mice developed large primary tumors as well as pulmonary metastases. During sacrifice, the lungs of the

mice were collected and metastasized cells (K7M2L1/lacZ) were isolated by digestion of the lung tissue using collagenase B (Roche, Mannheim, Germany) and subsequently selected in cell culture medium containing 800 µg/ml G418 (Invitrogen, Life Technologies, Carlsbad, CA, USA). The resulting K7M2L1/lacZ cells were then reinjected (i.t.) into BALB/c mice in order to obtain K7M2L2/lacZ cells in the same manner. After in vivo selection, K7M2L2/lacZ cells were transduced with an mCherry-containing plasmid (pQCIXH, containing a hygromycin selection marker), which was kindly provided by Prof. Markus Rudin (Institute of Biomedical Engineering, University and ETH Zurich, Zurich, Switzerland). After transduction, K7M2L2/lacZ/mCherry cells were selected in tissue culture medium with 1 mg/ml of G418 (Merck Millipore) and 400 µg/ml of hygromycin (Merck Millipore) to stably express lacZ and mCherry. A third round of transduction was performed using a pQCIXP plasmid containing the luciferase2 gene. The original plasmid was kindly provided by Dr. Geertje van der Horst (Leiden University, Leiden, The Netherlands). Ultimately, K7M2L2/lacZ/mCherry/luciferase2 cells (referred to as “K7M2L2” throughout the manuscript) were selected using growth medium supplemented with 1 mg/ml of G418, 400 µg/ml of hygromycin and 1 µg/ml of puromycin (Invitrogen).

3.2 K7M2L2 tumor inductions

Female, 8-week-old BALB/c mice (BALB/cAnNCrl; Charles River Laboratories, Sulzfeld, Germany) were maintained as described [22]. K7M2L2 cells were cultured below confluence and 1 x 105 K7M2L2 cells were injected into left tibias as described [25]. Upon signs of limping due to the tumor burden, 0.1 mg/kg of intraperitoneal (i.p.) Buprenorphine (Temgesic; Reckitt Benckiser, Berkshire, UK) was given twice daily. Subcutaneous (s.c.) tumors were induced by injecting 1 x 106 K7M2L2 cells into the left flank of mice and experimental metastases were induced through i.v. injection of 1 x 106 K7M2L2 cells via the tail vein. Body weight measurements were performed weekly. Animal care and experimental procedures were in accordance with the institutional guidelines and approved by the Ethics Committee of

the Veterinary Department, Canton of Zurich, Switzerland (license numbers 64/2013 and 160/2015).

3.3 Tumor monitoring and bone measurements

After xenografting, tumor growth in the hind limbs of the mice was monitored weekly using caliper measurements as described [26]. To determine tumor load in the lungs or detect remaining tumor cells at the site of amputation, luciferase activity was measured. Mice were anesthetized using 5% isoflurane (Forane; AbbVie, Inc., USA) and anesthesia was maintained with 2% isoflurane. XenoLight D-luciferin was injected via the mouse tail vein following the manufacturer's instructions (PerkinElmer, Waltham, MA, USA). Subsequently, luciferase activity was measured in an IVIS Lumina XR (Caliper Life Sciences, Inc., Hopkinton, MA, USA) and quantified with Living Image v4.4 software (Xenogen Corporation, Alameda, CA, USA).

Micro computed tomography (microCT) using a SkyScan1176 microCT system (Bruker, Billerica, MA, USA) was performed as described elsewhere [22]. Reconstituted images were segmented using CTAn v1.13.11.0 (Bruker, USA) to select regions of immature bone representing malignant bone formation. Immature bone volumes were determined in a region of interest starting from the distal end of the patella until the convergence of tibia and fibula. Volumes were calculated using the following formula: □cortical bone volume = bone volumetumor-limb - bone volumehealthy-limb.

3.4 Amputations

Amputations were performed under 2% isoflurane anesthesia with pre-operative 0.1 mg/kg of i.p. Buprenorphine, adapting the procedure described by Wolfe et al. [27]. In brief, the hip region of the tumor bearing leg was shaved and disinfected with 70% ethanol. A circumferential skin incision was made with small scissors at the plane of the femur/ proximal

to the primary tumor. After isolating the femoral artery, vein and nerve (see procedure described in [22]), the femoral artery and vein were concurrently ligated with sterile silk sutures (Fine Science Tools GmbH, Heidelberg, Germany) and cut proximal of the caudal femoral artery. The circumferential musculature was transected distal to the level of vessel ligation and separated from the femur. Using saw-toothed scissors, the femur was then transected proximal to the knee joint, at a level which ensured complete macroscopic removal of the primary tumor. Next, the sciatic nerve was identified and transected, followed by the remainder of the ischiocrural musculature to create a flap for subsequent closure. The remaining musculature was used to cover the distal end of the transected femur using 7-0 silk (Braun Melsungen, Melsungen, Germany). The skin was closed using 7-0 silk in an intermittent pattern. After surgery, the animals received i.p. 200 µl 0.9% NaCl to reestablish physiological fluid levels. Analgesia using 0.1 mg/kg of i.p. Buprenorphine was continued for 48 hours and followed by carprofen (Rimadyl; Pfizer, New York, NY, USA; final concentration: 0.067 mg/ml in the drinking water) given ad libitum for 5 days [28].

3.5 Determination of amount of circulating tumor cells and pulmonary metastases

After completion of the experiment, whole mouse blood was obtained during necropsy via cardiac puncture. Three healthy, untreated female BALB/c mice served as negative controls. Red blood cell lysis was performed using ammonium-chloride-potassium (ACK) lysis buffer and the remaining cells were divided into two parts. One part was briefly centrifuged at 1250 rpm for 3 min, dissolved in PBS and stored at -20°C for analysis of genomic DNA, while the second part was immediately analyzed using fluorescence-activated cell sorting (FACS). Genomic DNA was isolated using a QIAmp DNA blood Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Tumor-specific mCherry-DNA and glyceraldehyde 3-phosphate dehydrogenase (GAPDH)-DNA was amplified using quantitative PCR (input: 10 ng of genomic DNA). Primers were purchased from Microsynth (Balgach, Switzerland) and used at 200 nM. Primer sequences are listed in Supplementary Table S1. For quantitative PCR, amplification reactions were carried out using Power SYBR Green PCR master mix

(Applied Biosystems, Foster City, CA, USA) and the StepOne Plus Real- Time PCR system (Applied Biosystems). Relative amounts of genomic mCherry were determined using the 2-ΔΔCt method and normalized to the amount of murine GAPDH.

FACS was performed to detect mCherry-positive (mCherry+) circulating tumor cells. Following red blood cell lysis of whole blood, cells were suspended in PBS containing 0.5% FCS (Gibco by Thermo Fisher Scientific, Waltham, MA, USA). Fluorescent mCherry+ cells were counted and normalized to the input volume of whole blood using a FACS Aria III cellsorter (BD Biosciences, San Jose, CA, USA). Post mortem, pulmonary metastases were counted as described [26].

3.6 Experimental design

In total, three mayor studies were performed: 1) “comparative treatment study”: to compare injections of i.v. and i.a. F8-TNF without amputation of the primary tumor after establishment of palpable tumors (n ≥ 8). In the “comparative treatment” study, the time for metastasis development was limited due to the rapid increase in morbidity of the animals following primary tumor growth. To specifically test the anti-metastatic effect of F8-TNF against established pulmonary metastases, we employed an amputation model of metastatic osteosarcoma in a “pilot amputation study”. 2) “pilot amputation study”: to determine the metastatic load after hind limb amputation at specific tumor volumes (no primary tumor (PT): n = 3; small PT: n = 6; medium PT: n = 8; large PT: n = 9). 3) “combination treatment study”: to determine the effect of F8-TNF alone or in combination with DOX on the growth of spontaneous pulmonary metastases and potential local recurrences after amputation of the tumor-bearing hind limb (Group “vehicle”: n = 9; group “F8-TNF”: n = 9; group “DOX”: n = 8; group “Comb”(Combination; F8-TNF + DOX): n = 8). Mice that did not receive the planned doses were excluded from all analyses.

3.7 Drug infusions

F8-TNF was adjusted using PBS [18]. “Comparative treatment study”: Mice anesthetized with 2% isoflurane were kept warm on a heating mat throughout the procedure. Intravenous infusions were performed via the tail vein using a 30G needle attached to a polyethylene catheter (Portex; Smiths Medical, Inc., USA) under control of a syringe pump (Legato; WPI, Inc., USA). Intraarterial infusions were performed similarly as described [22]. Mice received each treatment (PBS or 2 µg of F8-TNF) three times. Substances were administered every 72 hours. In case of DOX, DOX (5 mg/kg, i.v.; Sandoz, Holzkirchen, Germany) treatment was administered only once, alone or prior to F8-TNF (2 µg per mouse, i.v.).

3.8 Immunohistology and immunofluorescence

Following excision, primary tumors and lungs were embedded in Tissue-Tek® O.C.T. Compound (Sakura Finetek, USA), frozen on dry ice and cut (8-10 µm) in a CM3050S cryotome (Leica Biosystems, Newcastle, UK). Frozen tissue sections were fixed in ice-cold acetone, blocked using 20% FCS (Gibco by Thermo Fisher Scientific) in PBS and stained. Immunofluorescence was applied on frozen sections to detect EDA (biotinylated F8; 1:100; as described [18]), CD31 (anti-PECAM-1 rabbit polyclonal antibody, sc-1506-R, Santa Cruz Biotechnology, USA; 1:200), CD4+ cells (rat anti-CD4, BioXCell, West Lebanon, NH, USA; 1:2000), CD8+ cells (rat anti-CD8a, BioXCell; 1:2000), CD19+ B cells (rat anti-CD19, BioXCell; 1:1000) and natural killer (NK) cells (rabbit anti-asialo GM1, Wako Pure Chemical Industries, Osaka, Japan; 1:3000) using the respective primary antibodies /small immunoproteins (SIP) diluted in 0.2% BSA (Sigma-Aldrich, St. Louis, MO, USA). Primary immunoproteins were detected with streptavidin-coupled Cy-3 (Sigma-Aldrich), goat anti-rabbit IgG-Alexa Fluor 647 (Life Technologies by Thermo Fisher Scientific), goat anti-rabbit IgG-Alexa Fluor 594 (Life Technologies) or goat anti-rat IgG-Alexa Fluor 594 (Life Technologies). Stained slides were mounted with fluorescent Shandon Immu-Mount (Thermo Fisher Scientific), images were taken with an Axio Observer Z1 (Zeiss, Oberkochen,

Germany) and analyzed using ImageJ v1.47 (U. S. National Institutes of Health, Bethesda, MD, USA).

3.9 Human osteosarcoma samples and ethics statement

The present study was approved by the local ethics committee (Ref. Nr. EK10/2007) and written informed consent was obtained from each subject or each subject's guardian. Primary human osteosarcoma tissue was stored at -80°C and embedded in Tissue-Tek® O.C.T. (Sakura) for sectioning and immunofluorescence.

3.10 Statistical Analysis

The results were given as mean ± standard error of the mean (SEM). Means of end point measurements were compared with one-way ANOVA or a Kruskal-Wallis test if the data was considered skewed (skewness >3). Means of time courses were compared by repeated two-way ANOVA unless otherwise stated. In case of ANOVA, Bonferroni posttests were used to determine statistical differences between individual groups (indicated in graphs) whereas Dunn's tests were used for skewed data. Dependent on the skewness of the data, Pearson or Spearman correlations were performed. All statistical tests were performed using Prism 5 v5.01 software (GraphPad Software, Inc., La Jolla, CA, USA), were 2-sided and $p<0.05$ was regarded as statistically significant.

4 Results

4.1 EDA is expressed in human osteosarcoma tissue

Immunofluorescence against EDA on various samples of primary human osteosarcoma demonstrated presence of EDA. Briefly, chemotherapy naïve osteosarcomas ("biopsy"; 5 of 6 (83%); EDA+ specimens of total specimens (percentage)) showed more intense fluorescent staining of EDA compared with chemotherapy treated ("resection"; 5 of 5 (100%)) samples

(Fig. 1). Metastases present in lung (“LMet”; 2 of 2 (100%)) or bone metastases (“BMet”; 2 of 2 (100%)) were all positive for EDA (Fig. 1).

4.2 Comparative treatment study

4.2.1 F8-TNF is well tolerated but lacks efficacy in primary tumors

After confirming the presence of EDA in human osteosarcoma, the effects of F8-TNF against osteosarcoma were evaluated dependent on the route of administration (i.a. versus i.v.) using the K7M2L2 osteosarcoma model. Efficacy of F8-TNF was determined by measuring the primary tumor volume using caliper. After completing three treatments, no significant differences in primary tumor volumes were observed (Fig. 2A). TNF-α belongs to the class of osteoclastogenic cytokines [29]. To see if F8-TNF treatment might impact bone remodeling, the formation of immature bone during tumor growth was evaluated. An increase in tumor-related immature bone production paralleling primary tumor volumes in the K7M2L2 model was observed (Pearson $r = 0.43$, $P = 0.007$). However, despite this correlation, and in line with the absence of changes in tumor growth, the immature bone produced by the tumors was not significantly affected by the F8-TNF treatment (Fig. 2B).

Furthermore, body weights of the mice were recorded to see if the treatment itself impacts the general health of the mice. Throughout the study, no significant differences were observed among the different treatment groups (Supplementary Fig. 1A). However, due to the tumor growth, a continuous loss of body weight was observed. Since two different routes of drug administration were compared, blood perfusion of the tumor-bearing limb was monitored to identify whether surgery had influenced downstream blood flow. After three rounds of i.a. or i.v. infusions, no significant differences in perfusion of the region of primary tumor growth were detected (Supplementary Fig. 1B).

4.2.2 Early metastatic growth is reduced by F8-TNF

In contrast to the absence of effects of F8-TNF against the primary tumor, a reduction in systemic disease was observed after F8-TNF treatment. Interestingly, dependent on the size of the pulmonary metastases, metastases were differentially affected. Large metastases (diameter > 0.5 mm; Fig. 2C) or medium-sized metastases (diameter 0.5 - 0.1 mm; Fig. 2D) were less affected by the treatment while F8-TNF significantly reduced the number of micrometastases (diameter < 0.1 mm; Fig. 2E). To see if this reduction in metastatic cells is also linked to a treatment-dependent reduction of circulating tumor cells (CTCs), the amount of genomic mCherry DNA was determined analyzing the cellular fraction of blood samples. Treatment with F8-TNF showed a trend towards reduced numbers of CTCs (Fig. 2F). The distribution pattern of the number of CTCs weakly correlated with the number of pulmonary micrometastases (Spearman r = 0.30, P = 0.127; data not shown). In addition to qPCR, the amount of CTCs after i.a. F8-TNF was determined using FACS in selected mice. Mice treated with i.a. F8-TNF had reduced numbers of mCherry+ tumor cells within the blood circulation (Supplementary Fig.S2).

4.2.3 F8-TNF activates immune cells in the lung parenchyma

Given the role of the immune system in controlling tumor progression, the numbers of various immune cells in the lung parenchyma were determined using immunofluorescence (Fig. 3A). Elevated numbers of CD4+ cells (Fig. 3B), CD8+ cells (Fig. 3C), and natural killer (NK) cells (Fig. 3D) were observed after F8-TNF treatment, irrespective of the route of drug administration.

4.3 EDA is expressed in experimental osteosarcomas and human osteosarcoma

To explain the differential efficacy of F8-TNF against the same cell line (metastases versus primary tumor), we investigated alterations in EDA-expression based on various microenvironments. Multiple K7M2L2-derived tumor tissues were analyzed using immunofluorescence (Fig. 4A). Although all experimental tumor samples were derived from

the same cell line, qualitative assessment showed only little expression or absence of EDA in intratibial primary tumors. In contrast, pulmonary metastases induced through i.v. injections or derived from a primary tumor were marked by high expression levels of EDA. Similarly, K7M2L2 cells s.c. injected yielded primary tumor volumes with increased levels of EDA compared with i.t. primary tumors. In line with the expression of EDA, s.c. primary tumors regressed (Fig. 4B) after F8-TNF treatment in contrast to intratibial (i.t.) primary tumors. Interestingly, varying expression levels and patterns of EDA (e.g. low versus high) were found between pulmonary metastases derived from the same K7M2L2 experimental primary tumor (data not shown).

4.4 Pilot amputation study

4.4.1 Large primary tumors are required for successful colonization of the lungs

Primary tumors (PT) were amputated at different sizes (no PT: <25 mm3; small PT: 27-61 mm3; medium PT: 102-250 mm3; large PT: 385-1500 mm3) and body weights as well as luciferase signals in the thoracic region were recorded. A significantly larger loss of body weights was detected in the group of large PT after amputation compared to the other groups (Fig. 5A). However, this difference disappeared three days later and no other differences in body weight loss were observed between the different groups. At the time of amputation, single tumor-bearing mice were sacrificed and the numbers of pulmonary metastases were determined. Large tumors correlated with higher numbers of pulmonary metastases (Fig. 5B). In addition, luciferase signals were measured in the thoracic region to determine metastatic burden and different patterns of tumor progression were observed (Supplementary Table S2). For instance, the luciferase activity remaining at the site of amputation spontaneously disappeared, whereas the tumor burden in the lung of the same mouse increased (Fig. 5C, large PT). Furthermore, continuous thoracic luciferase activity above tissue background (> 106 p/s) was only observed when large PTs were amputated (Fig. 5D).

4.5 Combination treatment study

4.5.1 F8-TNF does not prevent growth of established metastases

Next, we tested the efficacy of F8-TNF against already established pulmonary metastases. DOX, a standard drug in osteosarcoma chemotherapy, was shown to improve the anti-tumor effect of F8-TNF even further [18]. Therefore, we combined the use of DOX and F8-TNF to evaluate the individual and combined efficacies against systemic disease. The number of CTCs was evaluated using FACS of mCherry+ tumor cells. However, no significant differences in mean numbers of CTCs were observed (Fig. 6A). Mice treated with vehicle (range: 0-34 mCherry+ cells / ml) or DOX (0-11) only, showed the highest maximum number of CTCs compared to F8-TNF (0-5) or comb (0-2) treated animals.

Neither the absolute numbers of pulmonary metastases after sacrifice (Fig. 6B) nor the in vivo luciferase-activity measurements (Fig. 6C) detected significant differences between the various treatment groups. Nevertheless, a non-significant delay in growth of pulmonary metastases until the end of the treatment (= “9 d post treatment”) was observed with the combination treatment. All tested treatment regimens were well tolerated without causing a severe loss of body weight (Supplementary Fig. S3). Moreover, local soft tissue recurrences in the abdomen were detected in 33-38% of animals which have received limb amputation of a large primary tumor. However, no significant treatment-dependent differences in growth of local recurrences were observed (Supplementary Fig. S4).

4.6 Spleen size is increased by F8-TNF treatment but numbers of CD4+ and CD8+ cells are not affected

Enlarged spleens were observed in several immunocompetent mouse models of cancer and were associated with enhanced metastasis or increased granulocytosis [30-32]. Speculating that F8-TNF treatment would also influence the composition of lymphocytes or granulocytes in our osteosarcoma model, the lengths of spleens were measured. Significantly enlarged spleens were observed after F8-TNF treatment compared with vehicle- or DOX-treated mice

(Fig. 6A). The mean length of spleens of comb-treated mice was shorter than spleens of F8-TNF treated mice, without reaching significance. Similar to the “comparative treatment” study, we again investigated the immune infiltrate of the lungs. However, no significant differences among numbers of CD4+ (Fig. 6B) or CD8+ cells (Fig. 6C) in the lung parenchyma were identified.

5 Discussion

In this study, we studied the effects of targeted TNF-α in early and late stages of osteosarcoma biology. Here, we showed that F8-TNF targeted pulmonary metastases of osteosarcoma and reduced establishment of early-stage micrometastases, but was not able to significantly delay growth of already established pulmonary metastases. In addition, we could show that the route of administration (i.a. versus i.v.) of F8-TNF had no impact on drug efficacy, suggesting a targeting effect that is independent of temporary high local drug concentrations.

This study and others demonstrated the presence of EDA in primary human osteosarcoma tissues, rendering it a valid target for future therapeutic approaches against osteosarcoma [33]. However, our results demonstrated a differential expression of EDA in the K7M2L2 osteosarcoma model, with no expression in orthotopic primary tumors, whereas pulmonary metastases within the same animal expressed EDA strongly. As demonstrated by others, an osteoblast cell line and cells involved in human bone remodeling after a fracture strongly expressed EDA [34]. Thus, it is possible that disturbance due to i.t. injections in a bony microenvironment already induced expression of EDA, so that mostly EDA-negative primary i.t. tumors develop. To further investigate this observation, the same osteosarcoma cells were transplanted s.c. and mice were treated with F8-TNF. Not only EDA expression in subcutaneous tumors was observed but also strong anti-tumor effects of F8-TNF against s.c. K7M2L2 transplants were observed, supporting the results of Mortara et al. [35]. Moreover,

early lung tumors displayed a patchy expression of EDA in contrast to late lung tumors, where a vascular expression pattern of EDA was observed [36]. In line with these results, our study also demonstrated dynamic expression patterns of EDA in pulmonary metastases, ranging from patchy expression in early metastases to a patchy expression surrounding the pulmonary nodule while central EDA was mostly associated with the blood vessels. In summary, these results may indicate that the expression of EDA depends on the microenvironment and the developmental stage of the tumor. Therefore, the timing of administration of EDA-targeted chemokines should be considered in future efficacy studies.

It is already widely accepted that establishment of metastasis is a highly selective process. Thus, only a specific set of specialized tumor cells is capable of intravasation and distant organ colonization [37]. With respect to osteosarcoma, relatively little is known about CTCs, due to a lack of established markers. Nevertheless, it is known from other types of cancer, for instance breast cancer, that natural CTCs established metastases of a defined metastasis-related gene expression pattern that differed from the pattern derived from metastases established through systemic injection of tumor cells [38]. Especially expression of immunomodulatory molecules, such as the immune evasion signal CD47, might determine the success of CTCs to establish metastases in an immunocompetent host [39, 40]. In order to study the efficacy of an immunomodulatory drug such as F8-TNF, a model recapitulating the establishment of naturally- / immune-selected pulmonary metastases is required. The here presented study used an immunocompetent K7M2-derived model system which was already shown to properly mimic the different stages of human osteosarcoma progression (i.e. CTCs, development of pulmonary metastases) while at the same time permitting the use of basic osteosarcoma therapy (e.g. removal of the primary tumor), hence, it was considered clinically relevant [41].

In a first study, a reduction of micrometastatic disease and thus, preventive action of F8-TNF against seeding of pulmonary metastases was demonstrated. Previously, other studies demonstrated altered levels of immune cells which were linked to success of therapy against

osteosarcoma. For instance, an increase in CD8+ T cells [42] or increases in NK cells [43, 44] seem to be mediators of successful immunotherapy against osteosarcoma and its metastases. Likewise, our study demonstrated an increase in CD4+, CD8+ and NK cells in the lung parenchyma after F8-TNF treatment, which may provide an explanation for the reduced numbers of early pulmonary metastases. Especially early intraluminal metastatic seeds might be eliminated by an increase of NK cells in the lung parenchyma [45].

To study late metastatic disease, a model was required that allowed longer study times of pulmonary metastases. Due to ethical reasons, the presence of a too large primary tumor is considered as a drop-out criterion. Consequently, an amputation model not only provided longer study times of pulmonary metastases but was also a better representation of clinical late metastatic osteosarcoma, where the primary tumor was most frequently removed. Thus, the use of a model of spontaneous metastases is desired to fully demonstrate a drug's therapeutic value against late metastases [46]. Using such a model representing late stage osteosarcoma, we demonstrated that overt pulmonary metastases were not affected by TNF-F8 treatment. Even the addition of DOX to F8-TNF only slightly delayed further growth of established metastases. Interestingly, enlarged spleens were observed after F8-TNF therapy, yet only in the case of late metastatic disease. As demonstrated by others using fibrosarcoma or breast cancer models, enlarged spleens were correlated with increased numbers of MDSC [47] or neutrophils [48], respectively, both of which are indicative for an immunosuppressive environment [49]. These observations may suggest the presence or the adaptation of mechanisms helping metastasized tumor cells to successfully evade the immune system, which should be subject of further investigations.

This study showed that targeting of pulmonary metastasis using F8-TNF in osteosarcoma is feasible to control early stage metastasis, yet not sufficient to eradicate already established pulmonary metastases. Future studies should investigate the precise mechanisms of improved micrometastatic control of F8-TNF treatment. In addition, not only novel immune-targeted drugs influence the immune system but also chemotherapeutics used against

osteosarcoma affect cells of the immune system in many ways. For instance, multidrug chemotherapy can cause neutropenia [50], DOX can induce immunogenic cell death [9] and cisplatin can either increase or reduce the number of lymphocyte numbers dependent on its concentration [51]. Thus, future studies might also evaluate combinatorial treatments of F8-TNF together with conventional multidrug chemotherapy to identify optimal treatment schemes.

In conclusion, the present study demonstrated efficacy of F8-TNF against early metastatic disease irrespective of the route of administration. However, treatment with F8-TNF in combination with DOX had no significant inhibitory effect on late metastatic disease in a clinically relevant model of progressed osteosarcoma.

6 Acknowledgments

We would like to thank Philipp Probst and Teresa Hemmerle for their technical and administrative support.

7 List of abbreviations:

ACK: ammonium-chloride-potassium

BMet: bone metastasis

CTCs: circulating tumor cells

DOX: doxorubicin

EDA: extra-domain A

FACS: fluorescence-activated cell sorting

F8-TNF: EDA-targeted tumor necrosis factor alpha

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

i.p.: intraperitoneal

i.v.: intravenous

i.a.: intraarterial

LMet: lung metastasis

MDSC: myeloid-derived suppressor cell

PT: primary tumor

qPCR: quantitative polymerase chain reaction

s.c.: subcutaneous

SIP: small immuno-protein

TCI: tumor cell injection

TNF-α: tumor necrosis factor alpha

8 Ethics approval:

Animal care and experimental procedures were in accordance with the institutional guidelines and approved by the Ethics Committee of the Veterinary Department, Canton of Zurich, Switzerland (Licenses number 64/2013 and 160/2015). The patient samples were obtained after written informed consent and approval by the local ethics committee (Ref. Nr. EK10/2007).

9 Authors' Contributions:

Conceptualization: B. Robl, S.M. Botter, A. Boro, D. Neri, B. Fuchs

Methodology: B. Robl, B. Fuchs

Investigation: B. Robl, S.M. Botter, A. Boro, D. Meier

Project administration: B. Robl, A. Boro, S.M. Botter

Writing - original draft preparation: B. Robl

Writing - review and editing: S.M. Botter, D. Neri, B. Fuchs

Resources: D. Neri, B. Fuchs

Supervision: S.M. Botter, D. Neri, B. Fuchs

10 Titles and legends to figures

Figure 1. EDA is expressed in various human osteosarcoma tissues. Represantative examples of EDA expression in a biopsy (before chemotherapy), resection (after chemotherapy), bone metastasis (BMet) and lung metastasis (Lmet). 200X original magnification. Nuclei were counterstained with Hoechst (blue). All scale bars correspond to 100 µm.

Figure 2. F8-TNF (i.v. and i.a.) has no inhibitory effect on K7M2L2 primary tumor growth yet reduces numbers of small pulmonary metastases. (A) Tumor volumes determined by caliper ($n \geq 8$; repeated measures two-way ANOVA). Days of drug infusion are indicated by black arrow heads. (B) Immature bone volumes as determined by microCT scans before and after treatment ($n \geq 8$; repeated measures two-way ANOVA). Volumes of immature bone were analyzed both in control limbs and in tumor-bearing limbs. b: before treatment; a: after treatment; CtrL: control limb; TuL: tumor-bearing limb. Total number of superficial pulmonary metastases with (C) a diameter > 0.5 mm, (D) a diameter of 0.1 - 0.5 mm or (E) a diameter < 0.1 mm, determined after X-gal staining ($n \geq 5$). (F) Genomic mCherry normalized by GAPDH was analyzed using whole blood samples. Highest amount of mCherry was set as 100% and relative values are shown ($n \geq 6$).

Figure 3. Treatment with F8-TNF increases infiltration of immune cells in the lung. (A) Representative examples of immunofluorescence analysis of CD4+ T cells in the lung after each treatment (from top: i.v. vehicle; i.v. F8-TNF; i.a. vehicle; i.a. F8-TNF). Total numbers of

CD4+ ((B), n ≥ 4), CD8+ ((C), n ≥ 4) and NK cells ((D), n ≥ 3) determined in five high power fields (HPF) from lungs of individual mice. *p<0.05; **p<0.01; ***p<0.001.

Figure 4. EDA is expressed differently depending on the site of tumor growth. (A) Representative sections demonstrate site-dependent EDA (red) expression of K7M2L2 osteosarcoma cells. Intratibial primary tumor (i.t. PT): 100X original magnification; s.c. PT: 100X; intravenously injected lung metastasis (i.v. LMet): 100X; spontaneous LMet: 200X. All scale bars correspond to 100 μm. (B) Tumor volumes determined by caliper during F8-TNF treatment (n = 1-2).

Figure 5. Characterization of the osteosarcoma amputation model. (A) Changes in body weight prior to and after the amputation are indicated (n ≥ 3; repeated measures two-way ANOVA, **p<0.01). On the day of amputation (black arrow), body weights were measured before the amputation (d 29) and immediately after removal of the tumor-bearing limb (d 29.5). (B) Correlation between increasing primary tumor volume and metastases (n = 3; Pearson correlation). (C) Representative example of metastatic dynamics after amputation observed in the same mouse. After amputation, remaining tumor cells (black arrowhead) could disappear spontaneously (black arrowhead) or continue growing (not shown). i: 2 weeks (w) prior to amputation; ii: 10 min post amputation; iii: 1 month post amputation. (D) Development of luciferase activity in the lungs dependent on the size of the amputated PT (n = 3-9; repeated measures two-way ANOVA).

Figure 6. F8-TNF and DOX do not inhibit late stage metastatic osteosarcoma or affect lymphocytes in the lung. (A) CTCs analyzed using fluorescence-activated cell sorting of mCherry+ tumor cells detected in whole blood (n ≥ 8). (B) Quantification of lacZ+ osteosarcoma cells detected on the surface of the small lobe of the lung after X-gal staining postmortem (n ≥ 8). (C) Continuous in vivo monitoring of luciferase+ tumor cells in the thoracic region by measuring luminescent flux (n ≥ 8). Black arrowheads indicate administration of treatment. d: days. (D) Lengths of spleens were measured ex vivo after

sacrifice of the mice (n ≥ 8). Numbers of (E) CD4+ and (F) CD8+ cells in the lungs determined in five high power fields (HPF) from lungs of individual mice (n = 6).

11 References

1. Mirabello L, Troisi RJ, Savage SA. Osteosarcoma incidence and survival rates from 1973 to 2004: data from the Surveillance, Epidemiology, and End Results Program. Cancer. 2009;115(7):1531-43. Epub 2009/02/07. doi: 10.1002/cncr.24121. PubMed PMID: 19197972; PubMed Central PMCID: PMC2813207.

2. Howlader N NA, Krapcho M, Garshell J, Neyman N, Altekruse SF, Kosary CL, Yu M, Ruhl J, Tatalovich Z, Cho H, Mariotto A, Lewis DR, Chen HS, Feuer EJ, Cronin KA (eds). Cancer Epidemiology in Older Adolescents and Young Adults 15 to 29 Years of Age, Including SEER Incidence and Survival: 1975-2000. In: Institute NC, editor. NIH Pub No 06-5767. Bethesda, MD: SEER Cancer Statistics Review, 1975-2010; 2013.

3. Allison DC, Carney SC, Ahlmann ER, Hendifar A, Chawla S, Fedenko A, et al. A meta-analysis of osteosarcoma outcomes in the modern medical era. Sarcoma. 2012;2012:704872. Epub 2012/05/03. doi: 10.1155/2012/704872. PubMed PMID: 22550423; PubMed Central PMCID: PMC3329715.

4. Luetke A, Meyers PA, Lewis I, Juergens H. Osteosarcoma treatment - where do we stand? A state of the art review. Cancer treatment reviews. 2014;40(4):523-32. Epub 2013/12/19. doi: 10.1016/j.ctrv.2013.11.006. PubMed PMID: 24345772.

5. Jeys LM, Grimer RJ, Carter SR, Tillman RM, Abudu A. Post operative infection and increased survival in osteosarcoma patients: are they associated? Ann Surg Oncol. 2007;14(10):2887-95. Epub 2007/07/27. doi: 10.1245/s10434-007-9483-8. PubMed PMID: 17653803.

6. Fritzsching B, Fellenberg J, Moskovszky L, Sapi Z, Krenacs T, Machado I, et al. CD8/FOXP3-ratio in osteosarcoma microenvironment separates survivors from non-survivors: a multicenter validated retrospective study. Oncoimmunology. 2015;4(3):e990800. Epub 2015/05/08. doi: 10.4161/2162402X.2014.990800. PubMed PMID: 25949908; PubMed Central PMCID: PMC4404826.

7. Moore C, Eslin D, Levy A, Roberson J, Giusti V, Sutphin R. Prognostic significance of early lymphocyte recovery in pediatric osteosarcoma. Pediatr Blood Cancer. 2010;55(6):1096-102. Epub 2010/08/25. doi: 10.1002/pbc.22673. PubMed PMID: 20734401.

8. Liu T, Fang XC, Ding Z, Sun ZG, Sun LM, Wang YL. Pre-operative lymphocyte-to-monocyte ratio as a predictor of overall survival in patients suffering from osteosarcoma. FEBS Open Bio. 2015;5:682-7. Epub 2015/09/19. doi: 10.1016/j.fob.2015.08.002. PubMed PMID: 26380812; PubMed Central PMCID: PMC4556728.

9. Casares N, Pequignot MO, Tesniere A, Ghiringhelli F, Roux S, Chaput N, et al. Caspase-dependent immunogenicity of doxorubicin-induced tumor cell death. J Exp Med. 2005;202(12):1691-701. Epub 2005/12/21. doi: 10.1084/jem.20050915. PubMed PMID: 16365148; PubMed Central PMCID: PMC2212968.

10. Carswell EA, Old LJ, Kassel RL, Green S, Fiore N, Williamson B. An endotoxin-induced serum factor that causes necrosis of tumors. Proceedings of the National Academy

of Sciences of the United States of America. 1975;72(9):3666-70. Epub 1975/09/01. PubMed PMID: 1103152; PubMed Central PMCID: PMC433057.

11. Wiemann B, Starnes CO. Coley's toxins, tumor necrosis factor and cancer research: a historical perspective. Pharmacol Ther. 1994;64(3):529-64. Epub 1994/01/01. PubMed PMID: 7724661.

12. Gasparri A, Moro M, Curnis F, Sacchi A, Pagano S, Veglia F, et al. Tumor pretargeting with avidin improves the therapeutic index of biotinylated tumor necrosis factor alpha in mouse models. Cancer research. 1999;59(12):2917-23. Epub 1999/06/26. PubMed PMID: 10383155.

13. Palladino MA, Jr., Shalaby MR, Kramer SM, Ferraiolo BL, Baughman RA, Deleo AB, et al. Characterization of the antitumor activities of human tumor necrosis factor-alpha and the comparison with other cytokines: induction of tumor-specific immunity. J Immunol. 1987;138(11):4023-32. Epub 1987/06/01. PubMed PMID: 3295044.

14. Havell EA, Fiers W, North RJ. The antitumor function of tumor necrosis factor (TNF), I. Therapeutic action of TNF against an established murine sarcoma is indirect, immunologically dependent, and limited by severe toxicity. J Exp Med. 1988;167(3):1067-85. Epub 1988/03/01. PubMed PMID: 3351434; PubMed Central PMCID: PMC2188888.

15. Eggermont AM, Schraffordt Koops H, Lienard D, Kroon BB, van Geel AN, Hoekstra HJ, et al. Isolated limb perfusion with high-dose tumor necrosis factor-alpha in combination with interferon-gamma and melphalan for nonresectable extremity soft tissue sarcomas: a multicenter trial. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 1996;14(10):2653-65. Epub 1996/10/01. PubMed PMID: 8874324.

16. Tracey KJ, Cerami A. Tumor necrosis factor: a pleiotropic cytokine and therapeutic target. Annu Rev Med. 1994;45:491-503. Epub 1994/01/01. doi: 10.1146/annurev.med.45.1.491. PubMed PMID: 8198398.

17. Borsi L, Balza E, Carnemolla B, Sassi F, Castellani P, Berndt A, et al. Selective targeted delivery of TNFalpha to tumor blood vessels. Blood. 2003;102(13):4384-92. Epub 2003/08/23. doi: 10.1182/blood-2003-04-1039. PubMed PMID: 12933583.

18. Hemmerle T, Probst P, Giovannoni L, Green AJ, Meyer T, Neri D. The antibody-based targeted delivery of TNF in combination with doxorubicin eradicates sarcomas in mice and confers protective immunity. British journal of cancer. 2013;109(5):1206-13. Epub 2013/07/28. doi: 10.1038/bjc.2013.421. PubMed PMID: 23887603; PubMed Central PMCID: PMC3778281.

19. Curnis F, Sacchi A, Borgna L, Magni F, Gasparri A, Corti A. Enhancement of tumor necrosis factor alpha antitumor immunotherapeutic properties by targeted delivery to aminopeptidase N (CD13). Nature biotechnology. 2000;18(11):1185-90. Epub 2000/11/04. doi: 10.1038/81183. PubMed PMID: 11062439.

20. Lu L, Li ZJ, Li LF, Wu WK, Shen J, Zhang L, et al. Vascular-targeted TNFalpha improves tumor blood vessel function and enhances antitumor immunity and chemotherapy in colorectal cancer. J Control Release. 2015;210:134-46. Epub 2015/05/25. doi: 10.1016/j.jconrel.2015.05.282. PubMed PMID: 26003042.

21. Jaffe N, Knapp J, Chuang VP, Wallace S, Ayala A, Murray J, et al. Osteosarcoma: intra-arterial treatment of the primary tumor with cis-diammine-dichloroplatinum II (CDP). Angiographic, pathologic, and pharmacologic studies. Cancer. 1983;51(3):402-7. Epub 1983/02/01. PubMed PMID: 6571796.

22. Robl B, Botter SM, Pellegrini G, Neklyudova O, Fuchs B. Evaluation of intraarterial and intravenous cisplatin chemotherapy in the treatment of metastatic osteosarcoma using an orthotopic xenograft mouse model. J Exp Clin Cancer Res. 2016;35(1):113. Epub 2016/07/17. doi: 10.1186/s13046-016-0392-1. PubMed PMID: 27421768.

23. Sabile AA, Arlt MJ, Muff R, Bode B, Langsam B, Bertz J, et al. Cyr61 expression in Osteosarcoma indicates poor prognosis and promotes intratibial growth and lung metastasis in mice. J Bone Miner Res. 2011. Epub 2011/10/07. doi: 10.1002/jbmr.535. PubMed PMID: 21976359.

24. Fidler IJ, Naito S, Pathak S. Orthotopic implantation is essential for the selection, growth and metastasis of human renal cell cancer in nude mice [corrected]. Cancer metastasis reviews. 1990;9(2):149-65. Epub 1990/09/01. PubMed PMID: 2253314.

25. Husmann K, Arlt MJ, Jirkof P, Arras M, Born W, Fuchs B. Primary tumour growth in an orthotopic osteosarcoma mouse model is not influenced by analgesic treatment with buprenorphine and meloxicam. Lab Anim. 2015;49(4):284-93. Epub 2015/02/05. doi: 10.1177/0023677215570989. PubMed PMID: 25650386.

26. Arlt MJ, Banke IJ, Walters DK, Puskas GJ, Steinmann P, Muff R, et al. LacZ transgene expression in the subcutaneous Dunn/LM8 osteosarcoma mouse model allows for the identification of micrometastasis. J Orthop Res. 2011;29(6):938-46. Epub 2011/02/02. doi: 10.1002/jor.21304. PubMed PMID: 21284029.

27. Wolfe TD, Pillai SP, Hildreth BE, 3rd, Lanigan LG, Martin CK, Werbeck JL, et al. Effect of zoledronic acid and amputation on bone invasion and lung metastasis of canine osteosarcoma in nude mice. Clinical & experimental metastasis. 2011;28(4):377-89. Epub 2011/03/05. doi: 10.1007/s10585-011-9377-9. PubMed PMID: 21374084; PubMed Central PMCID: PMC4284437.

28. Ingrao JC, Johnson R, Tor E, Gu Y, Litman M, Turner PV. Aqueous stability and oral pharmacokinetics of meloxicam and carprofen in male C57BL/6 mice. Journal of the American Association for Laboratory Animal Science : JAALAS. 2013;52(5):553-9. Epub 2013/09/18. PubMed PMID: 24041210; PubMed Central PMCID: PMC3784660.

29. Nakashima T, Kobayashi Y, Yamasaki S, Kawakami A, Eguchi K, Sasaki H, et al. Protein expression and functional difference of membrane-bound and soluble receptor activator of NF-kappaB ligand: modulation of the expression by osteotropic factors and cytokines. Biochemical and biophysical research communications. 2000;275(3):768-75. Epub 2000/09/07. doi: 10.1006/bbrc.2000.3379. PubMed PMID: 10973797.

30. Cao M, Xu Y, Youn JI, Cabrera R, Zhang X, Gabrilovich D, et al. Kinase inhibitor Sorafenib modulates immunosuppressive cell populations in a murine liver cancer model. Lab Invest. 2011;91(4):598-608. Epub 2011/02/16. doi: 10.1038/labinvest.2010.205. PubMed PMID: 21321535; PubMed Central PMCID: PMC3711234.

31. DuPre SA, Hunter KW, Jr. Murine mammary carcinoma 4T1 induces a leukemoid reaction with splenomegaly: association with tumor-derived growth factors. Exp Mol Pathol. 2007;82(1):12-24. Epub 2006/08/22. doi: 10.1016/j.yexmp.2006.06.007. PubMed PMID: 16919266.

32. Sato N, Michaelides MC, Wallack MK. Characterization of tumorigenicity, mortality, metastasis, and splenomegaly of two cultured murine colon lines. Cancer research. 1981;41(6):2267-72. Epub 1981/06/01. PubMed PMID: 7237427.

33. Kilian O, Dahse R, Alt V, Zardi L, Rosenhahn J, Exner U, et al. Expression of EDA+ and EDB+ fibronectin splice variants in bone. Bone. 2004;35(6):1334-45. Epub 2004/12/14. doi: 10.1016/j.bone.2004.08.008. PubMed PMID: 15589214.

34. Kilian O, Dahse R, Alt V, Zardi L, Hentschel J, Schnettler R, et al. mRNA expression and protein distribution of fibronectin splice variants and high-molecular weight tenascin-C in different phases of human fracture healing. Calcif Tissue Int. 2008;83(2):101-11. Epub 2008/07/30. doi: 10.1007/s00223-008-9156-z. PubMed PMID: 18663401.

35. Mortara L, Orecchia P, Castellani P, Borsi L, Carnemolla B, Balza E. Schedule-dependent therapeutic efficacy of L19mTNF-alpha and melphalan combined with gemcitabine. Cancer Med. 2013;2(4):478-87. Epub 2013/10/25. doi: 10.1002/cam4.89. PubMed PMID: 24156020; PubMed Central PMCID: PMC3799282.

36. Wieckowski S, Hemmerle T, Prince SS, Schlienger BD, Hillinger S, Neri D, et al. Therapeutic efficacy of the F8-IL2 immunocytokine in a metastatic mouse model of lung adenocarcinoma. Lung cancer. 2015;88(1):9-15. Epub 2015/02/16. doi: 10.1016/j.lungcan.2015.01.019. PubMed PMID: 25682318.

37. Valastyan S, Weinberg RA. Tumor metastasis: molecular insights and evolving paradigms. Cell. 2011;147(2):275-92. Epub 2011/10/18. doi: 10.1016/j.cell.2011.09.024. PubMed PMID: 22000009; PubMed Central PMCID: PMC3261217.

38. Wagenblast E, Soto M, Gutierrez-Angel S, Hartl CA, Gable AL, Maceli AR, et al. A model of breast cancer heterogeneity reveals vascular mimicry as a driver of metastasis. Nature. 2015;520(7547):358-62. Epub 2015/04/10. doi: 10.1038/nature14403. PubMed PMID: 25855289; PubMed Central PMCID: PMC4634366.

39. Steinert G, Scholch S, Niemietz T, Iwata N, Garcia SA, Behrens B, et al. Immune escape and survival mechanisms in circulating tumor cells of colorectal cancer. Cancer research. 2014;74(6):1694-704. Epub 2014/03/07. doi: 10.1158/0008-5472.CAN-13-1885. PubMed PMID: 24599131.

40. Xu JF, Pan XH, Zhang SJ, Zhao C, Qiu BS, Gu HF, et al. CD47 blockade inhibits tumor progression human osteosarcoma in xenograft models. Oncotarget. 2015;6(27):23662-70. Epub 2015/06/21. doi: 10.18632/oncotarget.4282. PubMed PMID: 26093091; PubMed Central PMCID: PMC4695143.

41. Khanna C, Prehn J, Yeung C, Caylor J, Tsokos M, Helman L. An orthotopic model of murine osteosarcoma with clonally related variants differing in pulmonary metastatic potential. Clinical & experimental metastasis. 2000;18(3):261-71. Epub 2001/04/21. PubMed PMID: 11315100.

42. Yu Z, Geng J, Zhang M, Zhou Y, Fan Q, Chen J. Treatment of osteosarcoma with microwave thermal ablation to induce immunogenic cell death. Oncotarget. 2014;5(15):6526-39. Epub 2014/08/26. doi: 10.18632/oncotarget.2310. PubMed PMID: 25153727; PubMed Central PMCID: PMC4171648.

43. Fernandez L, Valentin J, Zalacain M, Leung W, Patino-Garcia A, Perez-Martinez A. Activated and expanded natural killer cells target osteosarcoma tumor initiating cells in an NKG2D-NKG2DL dependent manner. Cancer letters. 2015;368(1):54-63. Epub 2015/08/16. doi: 10.1016/j.canlet.2015.07.042. PubMed PMID: 26276724.

44. Guma SR, Lee DA, Yu L, Gordon N, Hughes D, Stewart J, et al. Natural killer cell therapy and aerosol interleukin-2 for the treatment of osteosarcoma lung metastasis. Pediatr Blood Cancer. 2014;61(4):618-26. Epub 2013/10/19. doi: 10.1002/pbc.24801. PubMed PMID: 24136885; PubMed Central PMCID: PMC4154381.

45. Spiegel A, Brooks MW, Houshyar S, Reinhardt F, Ardolino M, Fessler E, et al. Neutrophils Suppress Intraluminal NK Cell-Mediated Tumor Cell Clearance and Enhance Extravasation of Disseminated Carcinoma Cells. Cancer Discov. 2016;6(6):630-49. Epub 2016/04/14. doi: 10.1158/2159-8290.CD-15-1157. PubMed PMID: 27072748; PubMed Central PMCID: PMC4918202.

46. Khanna C, Fan TM, Gorlick R, Helman LJ, Kleinerman ES, Adamson PC, et al. Toward a drug development path that targets metastatic progression in osteosarcoma. Clinical cancer research : an official journal of the American Association for Cancer Research. 2014;20(16):4200-9. Epub 2014/05/08. doi: 10.1158/1078-0432.CCR-13-2574. PubMed PMID: 24803583; PubMed Central PMCID: PMC4134738.

47. Marigo I, Bosio E, Solito S, Mesa C, Fernandez A, Dolcetti L, et al. Tumor-induced tolerance and immune suppression depend on the C/EBPbeta transcription factor. Immunity. 2010;32(6):790-802. Epub 2010/07/08. doi: 10.1016/j.immuni.2010.05.010. PubMed PMID: 20605485.

48. Coffelt SB, Kersten K, Doornebal CW, Weiden J, Vrijland K, Hau CS, et al. IL-17-producing gammadelta T cells and neutrophils conspire to promote breast cancer metastasis. Nature. 2015;522(7556):345-8. Epub 2015/03/31. doi: 10.1038/nature14282. PubMed PMID: 25822788; PubMed Central PMCID: PMC4475637.

49. Cortez-Retamozo V, Etzrodt M, Newton A, Rauch PJ, Chudnovskiy A, Berger C, et al. Origins of tumor-associated macrophages and neutrophils. Proceedings of the National Academy of Sciences of the United States of America. 2012;109(7):2491-6. Epub 2012/02/07. doi: 10.1073/pnas.1113744109. PubMed PMID: 22308361; PubMed Central PMCID: PMC3289379.

50. Whelan JS, Bielack SS, Marina N, Smeland S, Jovic G, Hook JM, et al. EURAMOS-1, an international randomised study for osteosarcoma: results from pre-randomisation treatment. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO. 2015;26(2):407-14. Epub 2014/11/26. doi: 10.1093/annonc/mdu526. PubMed PMID: 25421877; PubMed Central PMCID: PMC4304379.

51. Crum ED. Effect of cisplatin upon expression of in vivo immune tumor resistance. Cancer Immunol Immunother. 1993;36(1):18-24. Epub 1993/01/01. PubMed PMID: 8422664.

Supplementary Table S1. Primer sequences		
	Forward	Reverse
mCherry (genomic)	5‘-CAAGCTGAAGGTGACCAAGG	5’-CTCGAAGTTCATCACGCGCT
Murine GAPDH (genomic)	5‘-TGAAATGTGCACGCACCA	5‘-GGGAAGCAGCATTCAGGT

Supplementary Table S2. Development of pulmonary metastases and recurrences in dependence of tumor volume at amputation

	Number of mice (%)				
Group	**LMet at amputation**	**Luciferase signal at amputation site**	**LMet after 2 months**	**Abdominal recurrence after 2 months**	**Total**
No detectable PT	0 (0)	0 (0)	0 (0)	0 (0)	3
Small PT	2 (22)	0 (0)	0 (0)	0 (0)	6
Medium PT	0 (0)	0 (0)	0 (0)	0 (0)	6
Large PT	6 (66)	3 (33)	5 (56)	2 (22)	9

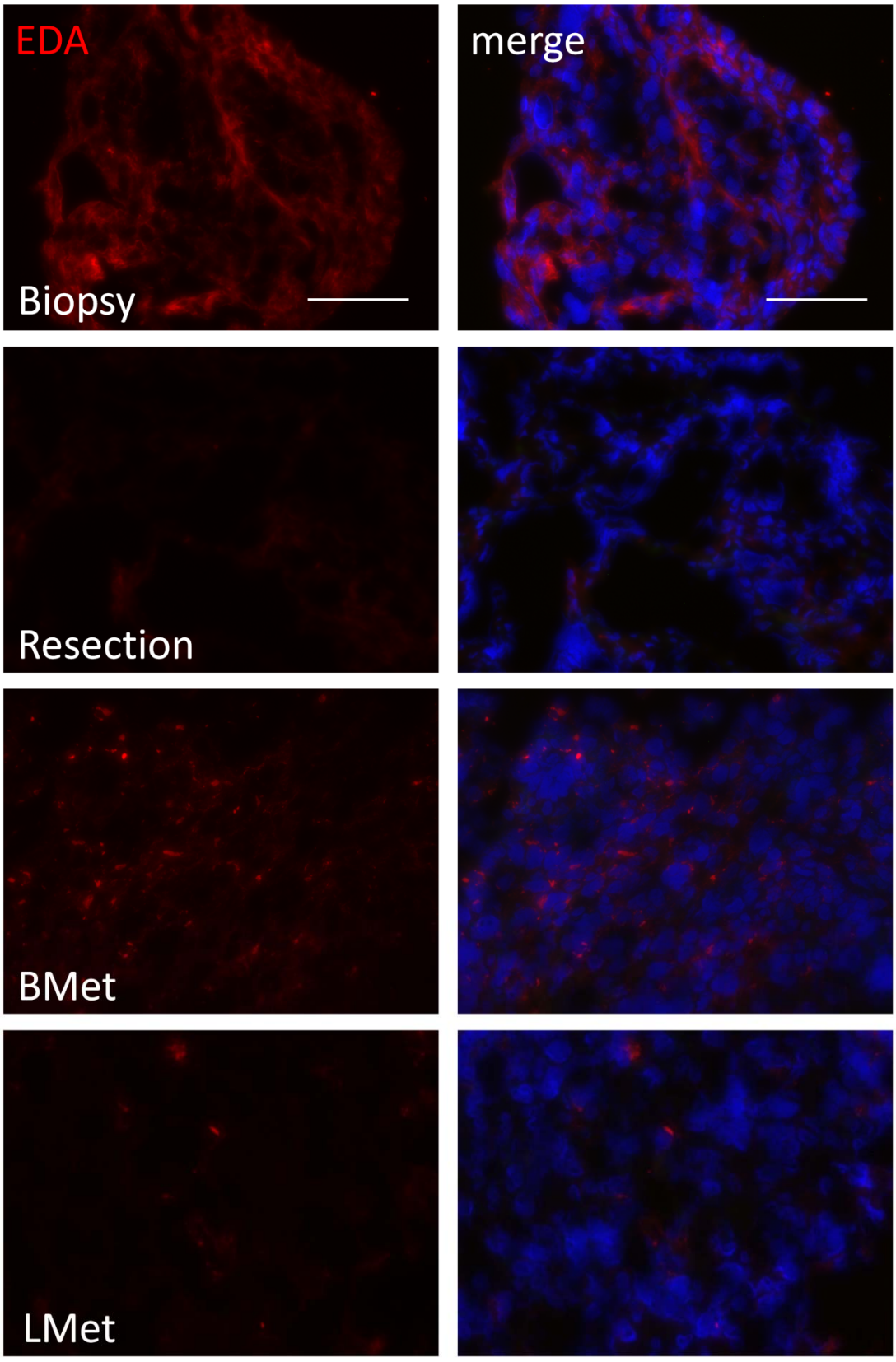


Figure 1

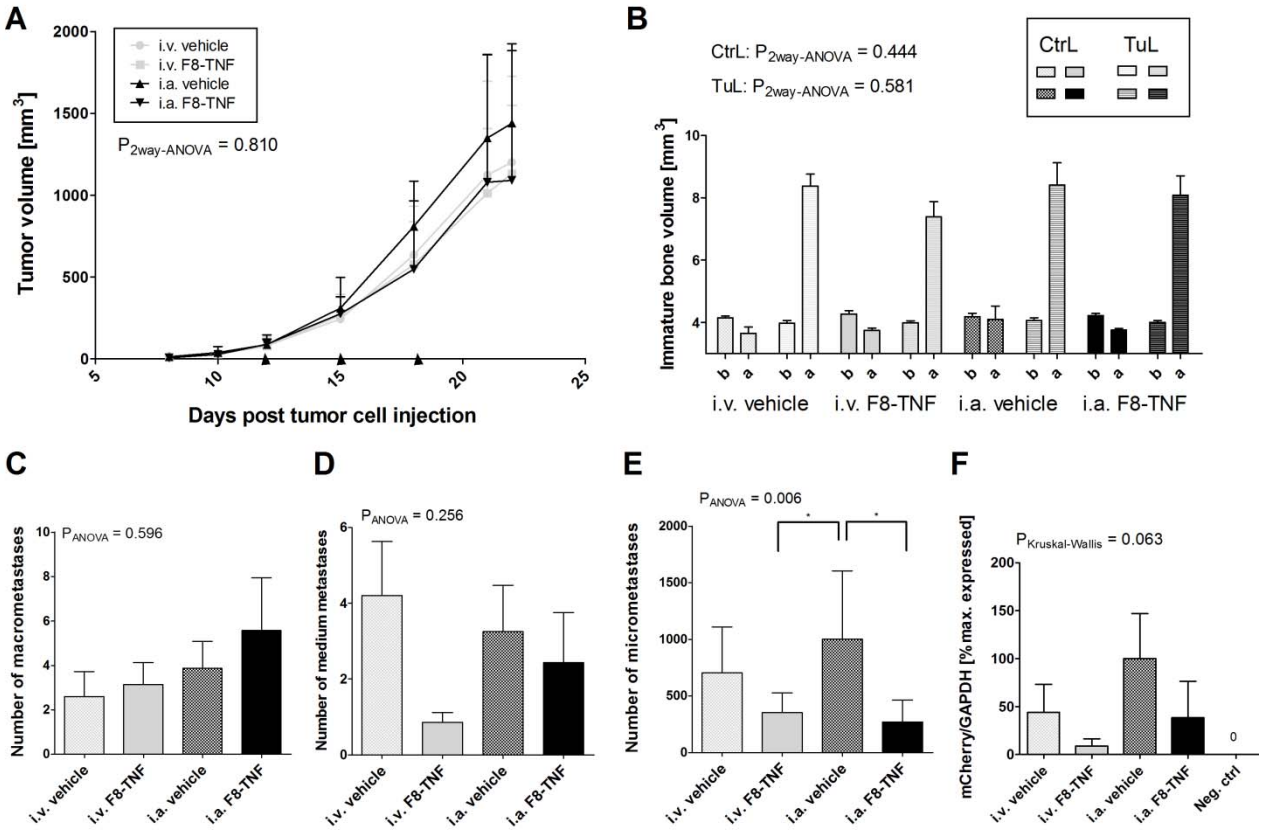

A
i.v. vehicle
i.v. F8-TNF
i.a. vehicle
i.a. F8-TNF
P2way-ANOVA = 0.810
Tumor volume [mm3]
Days post tumor cell injection
B
CtrL: P2way-ANOVA = 0.444
TuL: P2way-ANOVA = 0.581
CtrL
TuL
Immature bone volume [mm3]
b a b a b a b a b a b a b a b a
i.v. vehicle
i.v. F8-TNF
i.a. vehicle
i.a. F8-TNF
C
PANOVA = 0.596
Number of macrometastases
i.v. vehicle
i.v. F8-TNF
i.a. vehicle
i.a. F8-TNF
D
PANOVA = 0.256
Number of medium metastases
i.v. vehicle
i.v. F8-TNF
i.a. vehicle
i.a. F8-TNF
E
PANOVA = 0.006
Number of micrometastases
i.v. vehicle
i.v. F8-TNF
i.a. vehicle
i.a. F8-TNF
F
PKruskal-Wallis = 0.063
mCherry/GAPDH [% max. expressed]
i.v. vehicle
i.v. F8-TNF
i.a. vehicle
i.a. F8-TNF
Neg. ctrl

Figure 2

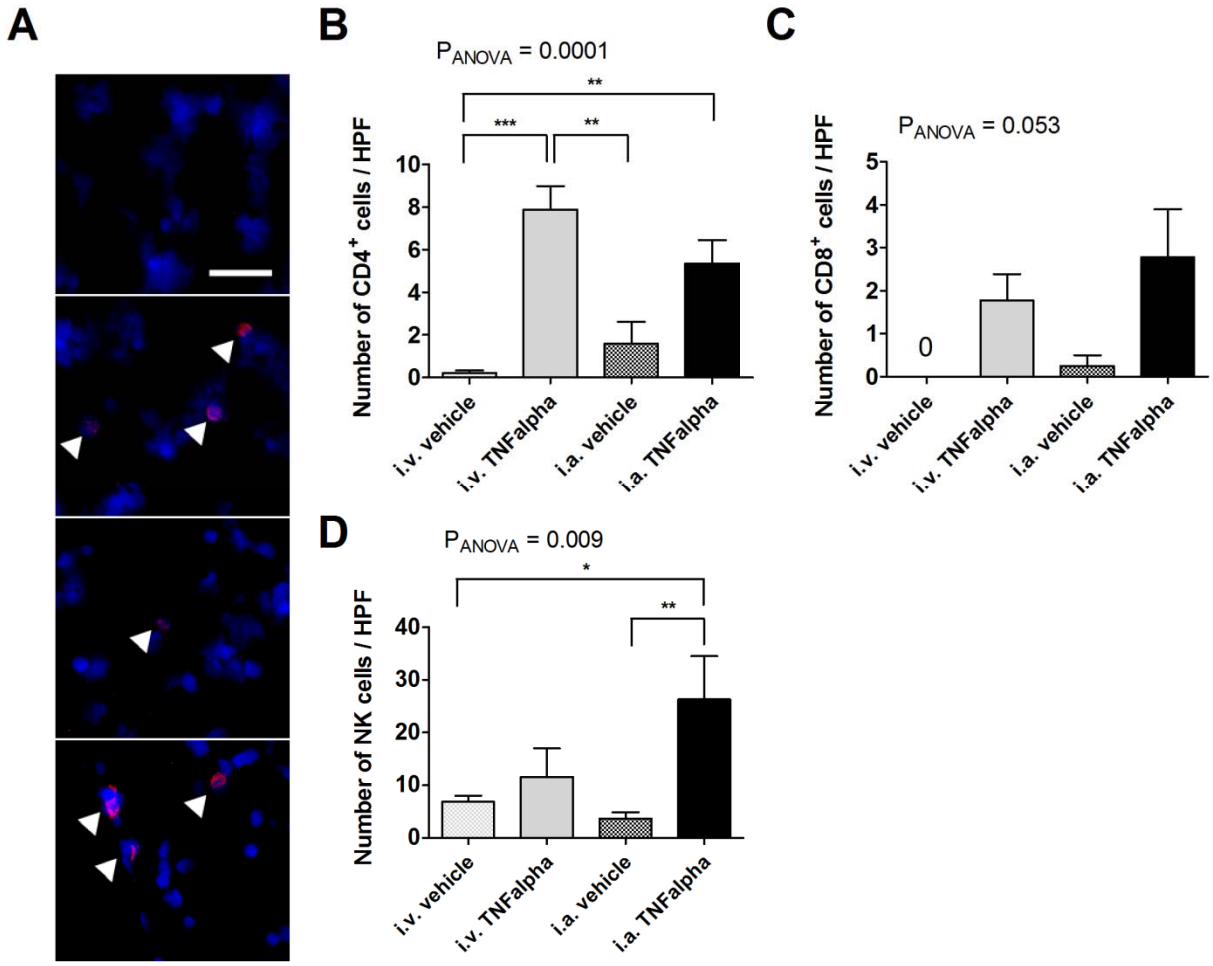

A
B
C
D
P_{ANOVA} = 0.0001
Number of $CD4^+$ cells / HPF
P_{ANOVA} = 0.053
Number of $CD8^+$ cells / HPF
0
P_{ANOVA} = 0.009
Number of NK cells / HPF
i.v. vehicle
i.v. TNFalpha
i.a. vehicle
i.a. TNFalpha

Figure 3

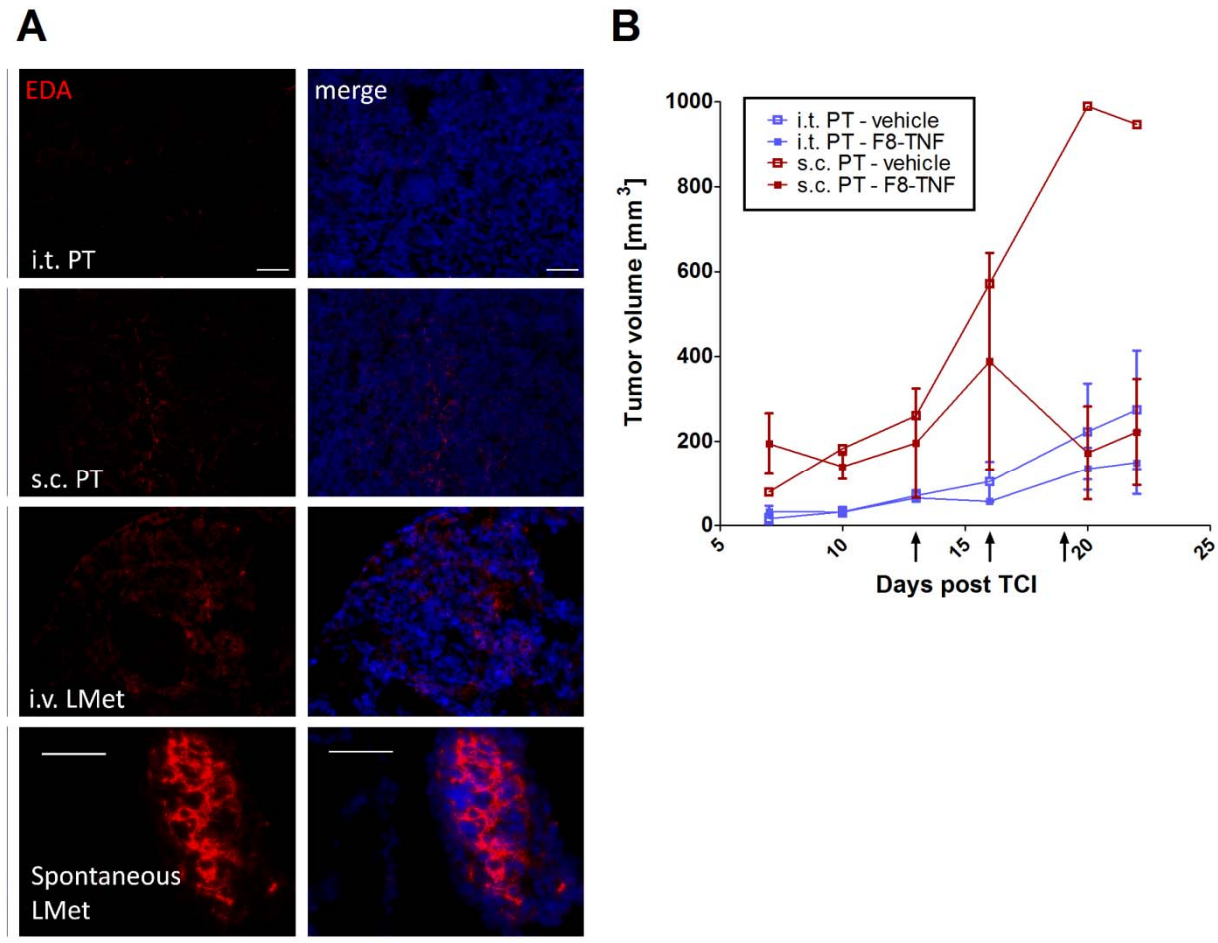

A
EDA
merge
i.t. PT
s.c. PT
i.v. LMet
Spontaneous LMet
B
Tumor volume [mm3]
1000
800
600
400
200
0
i.t. PT - vehicle
i.t. PT - F8-TNF
s.c. PT - vehicle
s.c. PT - F8-TNF
5
10
15
20
25
Days post TCI

Figure 4

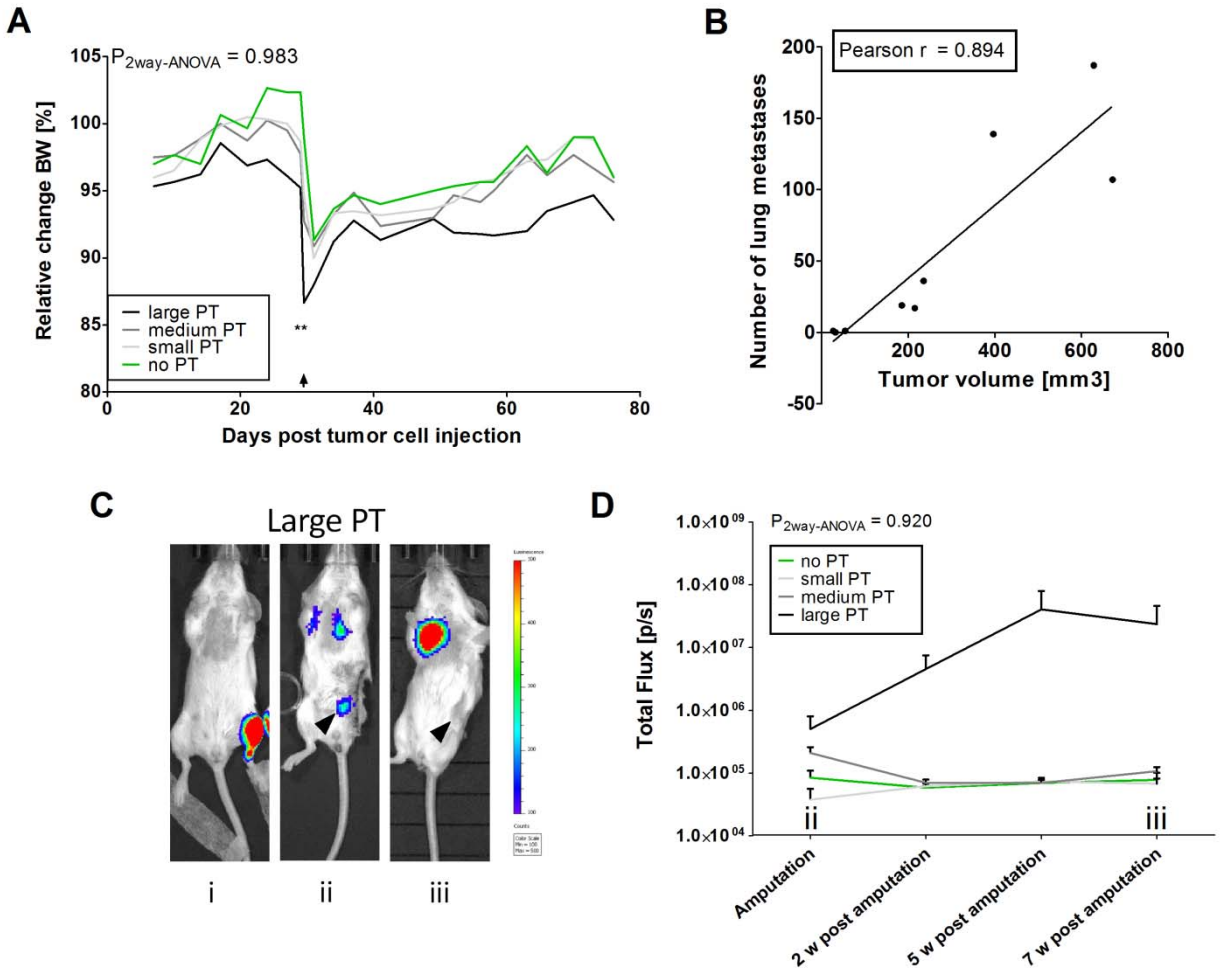

A
P2way-ANOVA = 0.983
Relative change BW [%]
large PT
medium PT
small PT
no PT
**
Days post tumor cell injection
B
Pearson r = 0.894
Number of lung metastases
Tumor volume [mm3]
C
Large PT
i
ii
iii
D
P2way-ANOVA = 0.920
no PT
small PT
medium PT
large PT
Total Flux [p/s]
Amputation
2 w post amputation
5 w post amputation
7 w post amputation

Figure 5

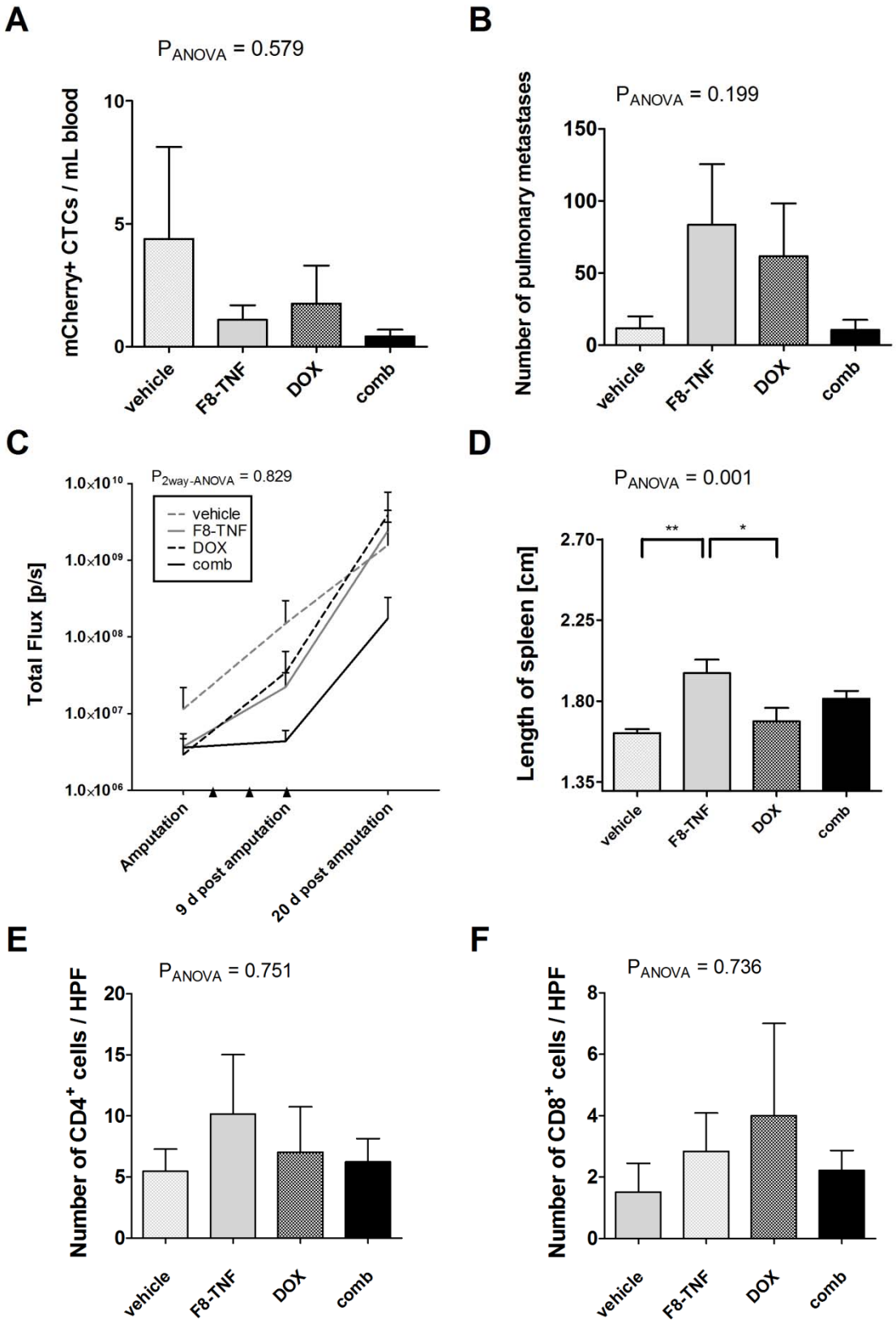
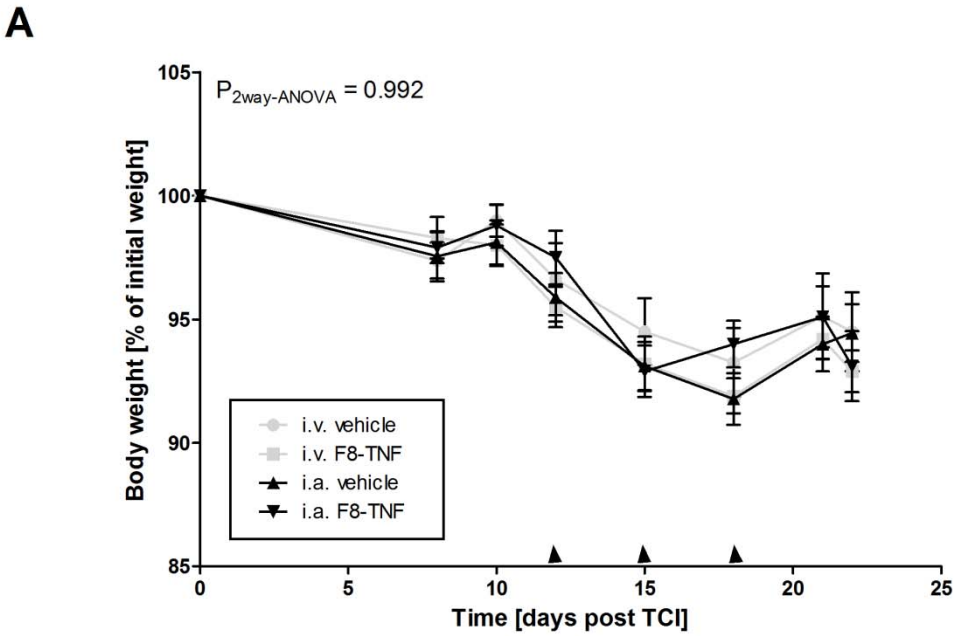
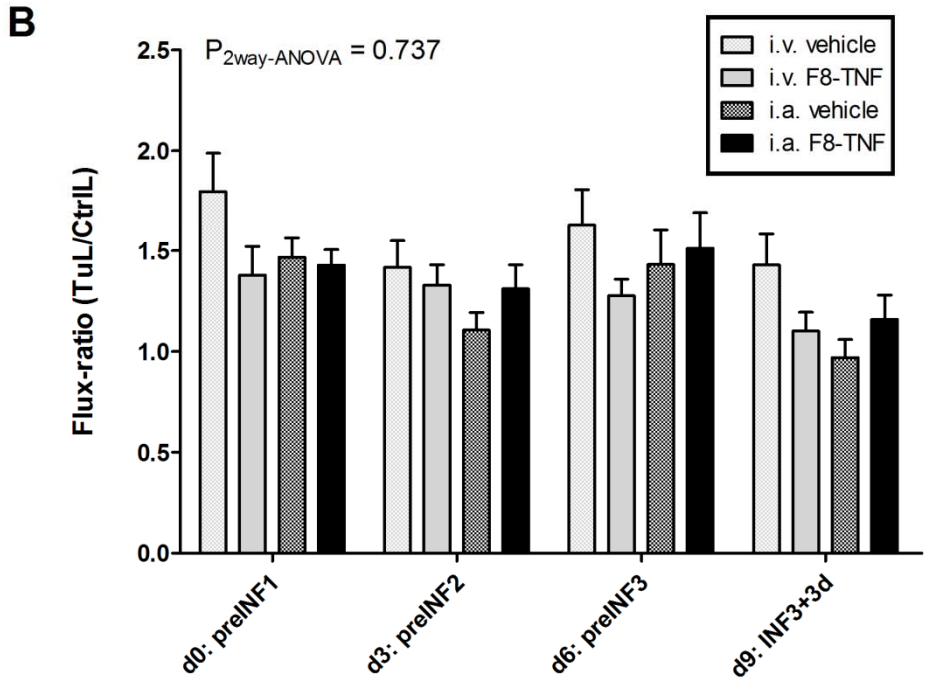

A
P ANOVA = 0.579
mCherry+ CTCs / mL blood
10
5
0
vehicle
F8-TNF
DOX
comb
B
P ANOVA = 0.199
Number of pulmonary metastases
150
100
50
0
vehicle
F8-TNF
DOX
comb
C
P 2way-ANOVA = 0.829
Total Flux [p/s]
1.0x10 10
1.0x10 09
1.0x10 08
1.0x10 07
1.0x10 06
vehicle
F8-TNF
DOX
comb
Amputation
9 d post amputation
20 d post amputation
D
P ANOVA = 0.001
Length of spleen [cm]
2.70
2.25
1.80
1.35
**
*
vehicle
F8-TNF
DOX
comb
E
P ANOVA = 0.751
Number of CD4+ cells / HPF
20
15
10
5
0
vehicle
F8-TNF
DOX
comb
F
P ANOVA = 0.736
Number of CD8+ cells / HPF
8
6
4
2
0
vehicle
F8-TNF
DOX
comb

Figure 6

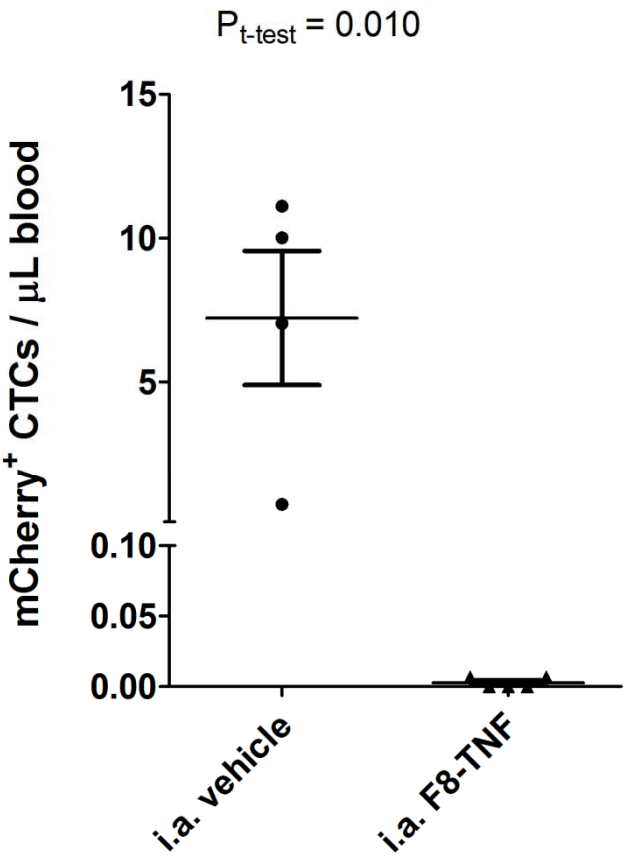


(A) Changes in body weight as an indicator for general health of the mice (n >= 8; repeated measures two-way ANOVA).



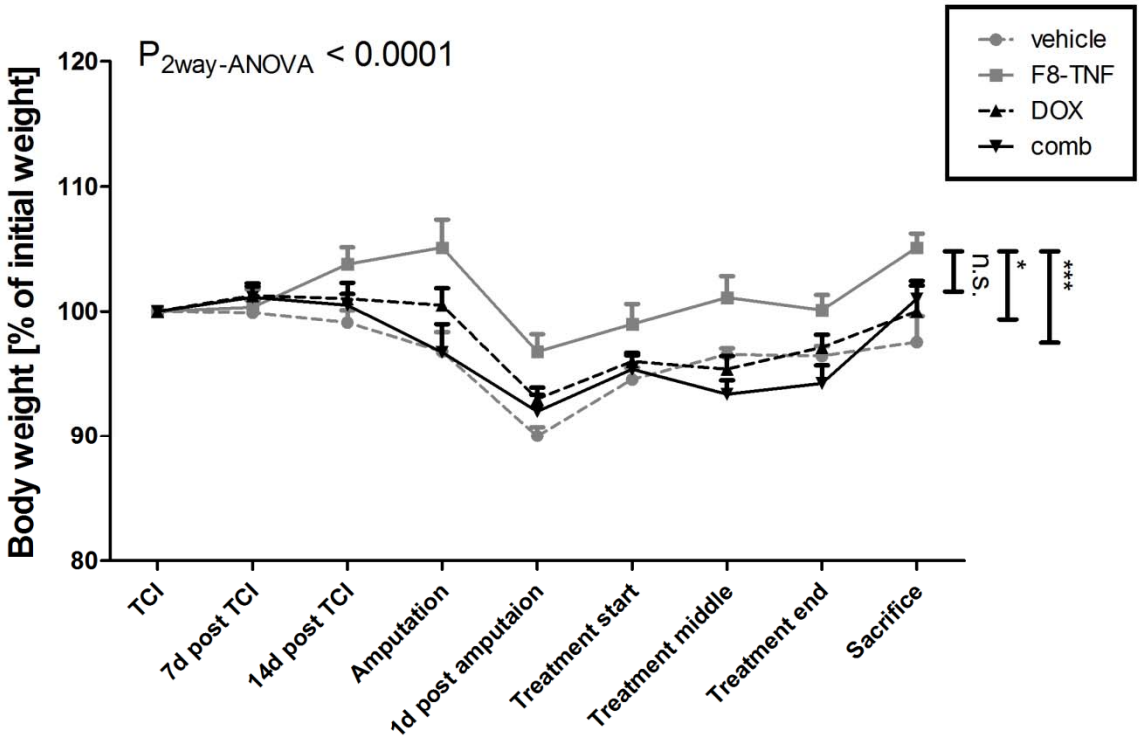
(B) Flux-ratios as indicators of perfusion of the tumor-bearing hind limb (n >= 8; repeated measures two-way ANOVA). CtrL: control limb; TuL: tumor-bearing limb;

Figure S1



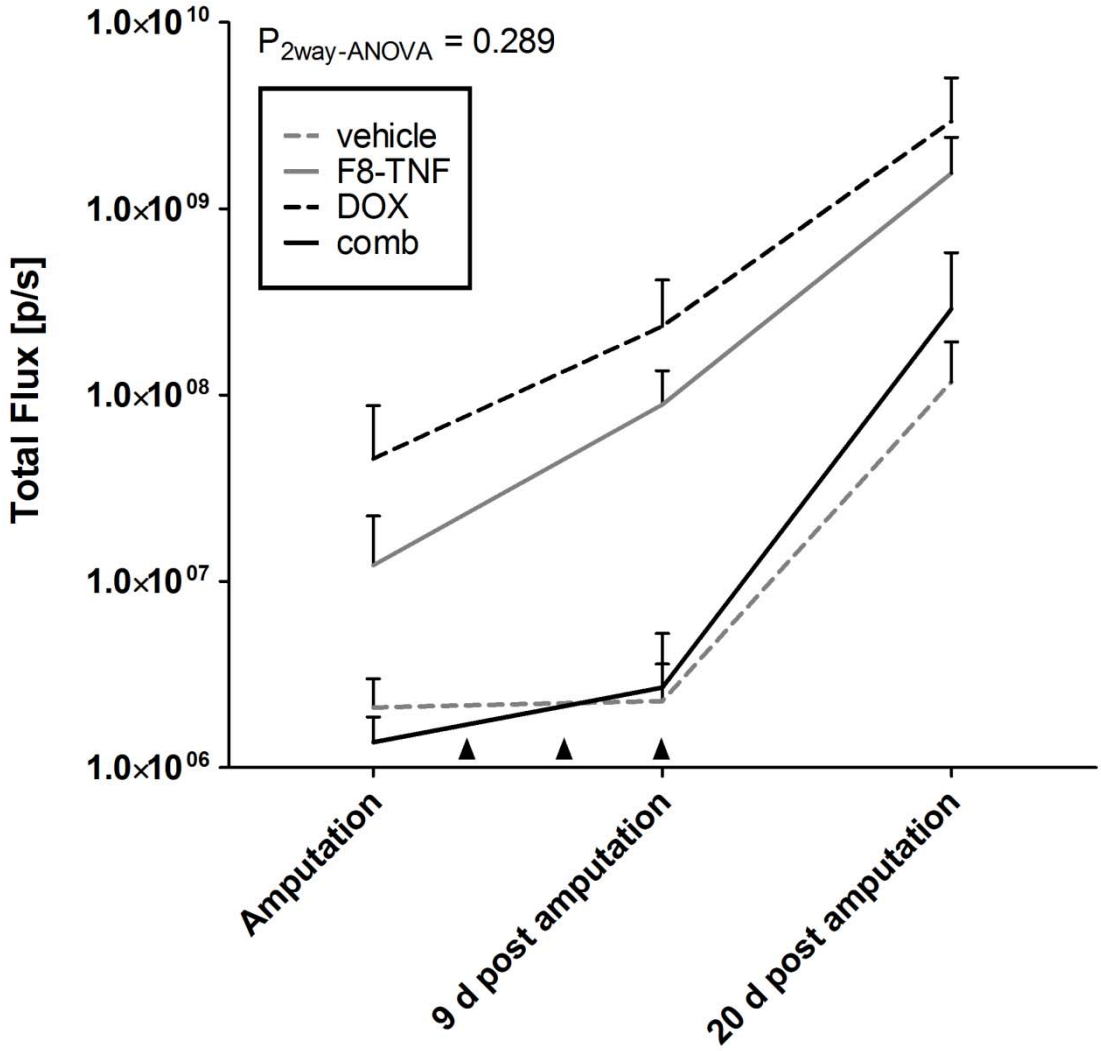
FACS analysis of mCherry+ CTCs in whole blood of selected mice at the end of the study. (n = 4-5; t-test).

Figure S2



Changes of relative body weights throughout the combination treatment study (n >= 8; repeated measures two-way ANOVA). TCI: tumor cell injection; d: days.

Figure S3



Changes of abdominal luciferase activity throughout the combination treatment study (n >= 8; repeated measures two-way ANOVA). Black arrowheads indicate administration of treatment. d: days.

Figure S4

12 Supplementary methods

Tumor perfusion measurements

To monitor tumor perfusion, laser speckle contrast imaging of the hind limbs was conducted using a moorFLPI Full-Field Perfusion Imager (Moor instruments Ltd., Devon, UK). Flux-ratios (flux-ratio= $Flux_{tumor\text{-}bearing\ limb}$ / $Flux_{contralateral\ limb}$, similar to as described by Robl et al..

Robl B, Botter SM, Pellegrini G, Neklyudova O, Fuchs B. Evaluation of intraarterial and intravenous cisplatin chemotherapy in the treatment of metastatic osteosarcoma using an orthotopic xenograft mouse model. J Exp Clin Cancer Res. 2016;35(1):113. Epub 2016/07/17. doi: 10.1186/s13046-016-0392-1. PubMed PMID: 27421768.

8 Conclusion and Outlook

Despite advances in multi-modal treatment, survival rates of metastatic osteosarcoma patients remain at a low 20-30% since the start of the clinical documentations of osteosarcoma. Nevertheless, surgery combined with multidrug chemotherapy increased survival rates of patients with localized disease to 60%, yet remained at this level for almost four decades. These numbers clearly highlight the need for improved treatment strategies for a rare disease such as osteosarcoma. Hence, a better understanding of the disease at many stages is required to improve osteosarcoma therapy. To achieve this goal, sophisticated preclinical models as well as analysis of clinical samples are required in order to better understand the influence of modern chemotherapy against osteosarcoma.

Until now, mostly untreated (naïve) patient material is used for the investigation of putative biomarkers for osteosarcoma under the valid presumption that chemotherapy influences the protein expression pattern of tumor cells. Thus, a biomarker detected in untreated tissue might have a different prognostic value if the same biomarker is studied in already treated tissue. Our results demonstrated that the use of chemotherapy-treated material, which is available at larger quantities compared with biopsied material, is feasible under certain conditions (e.g. analysis of tissue derived from grade 2 to grade 6 responders) for studying molecular markers in primary human osteosarcoma tissue. Surprisingly, the presence of cytP16 correlated with a poor response and poor prognosis if treated osteosarcoma samples were studied. This is contrasting with the prognostic value of nuclear p16 being indicative for improved survival if studied in untreated chemotherapy-naïve tissue. Likely, chemotherapy altered osteosarcoma cells by inducing a dysregulation of the p16 protein, changing its prognostic value. Given the altered subcellular localization of p16 after chemotherapy, the protein itself (238), the function of nuclear-cytoplasmic shuttling (240) or the structure of the nuclear pore complex (243) might be altered in surviving tumor cells after multidrug chemotherapy. Nevertheless, further studies are required to more completely decipher the role of cytP16 in the resistance mechanisms of osteosarcoma cells against multidrug

chemotherapy. Our studies provide a basis for future studies to specifically study resistance mechanisms of osteosarcoma against standard chemotherapy, for instance through mechanistic studies using the herein described cytP16^{+} osteosarcoma cell lines. It will be interesting to see, if the occurrence of cytP16 in these cell lines has a common origin. Studying of potential findings in treated primary osteosarcoma tissues might confer clinical relevance as well as provide a rational for further testing of additives to standard chemotherapy. Ultimately, the so gained knowledge could improve combination therapies through specific tailoring of standard chemotherapy to counteract developing mechanisms of resistance.

Another means of potentially improving current chemotherapy is the local administration of chemotherapy. Here presented results demonstrated a clear advantage of i.a. CDDP in terms of tumor control compared with equivalent concentrations of i.v. CDDP. Similar to CDDP, other small molecules used in anti-cancer therapy could achieve a much larger effect with respect to tumor control if administered via the tumor feeding artery compared with systemic administration. Our study demonstrated a user-friendly i.a. approach that could economically be used by many other researchers conducting mouse studies. Using i.a. infusions, much higher local doses could be achieved without compromising systemic drug concentrations. Despite the advantages of i.a. access in osteosarcomas of the extremities, future studies might also evaluate i.a. drug administrations against other solid cancers with relatively easy access to a tumor-feeding artery. Even in the case of targeted therapy (i.e. small molecule inhibitors against a specific target), i.a. infusions might achieve a more complete perfusion of poorly vascularized regions of a tumor and thus, might reduce the frequency of drug resistances occurring in those regions. Especially anti-angiogenic small molecules such as pazopanib or sunitinib might be interesting candidates for i.a. administration. First, similar to cytotoxic chemotherapy, systemic administration of sunitinib is limited by severe side effects (244). Secondly, tumor control by i.a. CDDP in our study was, at least partially, achieved through anti-angiogenic effects. Future therapy studies might

evaluate small molecular drugs in a route-dependent manner to optimally titrate the dose to be received by the tumor while optimally sparing sensitive healthy tissues, similar to approaches used in radiotherapy. Finally, more effective first-line therapy against the primary tumor might also decrease the development of metastases. Thus, we emphasize the evaluation of i.a. administrations of novel as well as already known drugs against solid cancers using a similar preclinical model system as presented in course of this work.

Nevertheless, before clinical use of i.a. chemotherapy against osteosarcoma might be appealing again, a future preclinical study should evaluate the efficacy of i.a. administration of the already used multidrug regimen (i.e. MAP) and compare it to i.v. administrations in a clinically relevant experimental model. Especially the evaluation of the long term effects such as development and growth of metastases or survival of the model animals in dependence of the route of standard chemotherapy administration will be important. The model systems described in this work fulfill these criteria and therefore lay a foundation for future preclinical studies assessing the administration of various combined chemotherapy protocols against osteosarcoma in a route-dependent manner.

In osteosarcoma, pulmonary metastases are still the most common cause of disease-related death. Therefore, a potential increase in osteosarcoma patient survival implies the effective treatment of systemic spread and metastatic disease. Due to the fact that surgical removal of metastases is limited by the used detection methods and the concomitant removal of vital tissues, alternative metastases-targeted approaches are required. At best, eradication of systemic disease is achieved through a metastasis-targeted activation of the immune system and the generation of immune memory to avoid regrowth of systemic cancer. To this end, a novel immunostimulatory compound (F8-TNF) as targeted treatment against pulmonary metastases was evaluated. Despite efficacy against early pulmonary metastases, F8-TNF did not reduce the growth of late, established pulmonary metastases. Thus, our results ask for a more appropriate or more efficacious treatment. However, combination with immunostimulatory DOX (245) only slightly improved therapeutic efficacy of F8-TNF against

established metastatic disease. The fact that both drugs are activators of the immune system, suggest the presence of an immunosuppressive environment being created by late pulmonary metastases. Although our results support the use of other cytokines than TNF-α to be used against metastatic osteosarcoma, the principle of guiding cytokines using an EDA-targeting moiety (i.e. F8) to pulmonary metastases might still be successfully applied against osteosarcoma as demonstrated by the presence of EDA in various human tissue specimens. Therefore, subsequent studies might evaluate other EDA-targeted cytokines and drug combinations against metastatic osteosarcoma. A promising approach could be the combination of an EDA-targeted immunostimulatory cytokine with immune blockade inhibitors (e.g. ipilimumab or anti-PD1/PDL1 antibodies) to guide therapy towards established metastatic disease. Anti-osteoclastogenic cytokines such as IFN-γ and IL-13 or a potent stimulator of NK cells such as IL-2 might also be more appropriate than pro-osteoclastogenic TNF-α in treating osteogenic pulmonary metastases (246), considering the interdependence between osteosarcoma, immune cells and osteoclast development.

In conclusion, the current successful multi-modal treatment approach as well as new therapeutic approaches of osteosarcoma therapy should be studied using proper preclinical models supported by the use of clinical osteosarcoma specimens in order to improve survival rates of patients with metastatic osteosarcoma. Using the here presented preclinical toolbox (i.e. i.v. versus i.a., surgical removal of the primary tumor, targeted cytokines, standard chemotherapeutics, *in vivo* monitoring of metastatic disease), a multitude of combinatorial treatment regimens can already be evaluated in a preclinical yet clinically relevant setting to precisely study the mechanisms of action of various drug candidates. A more complete understanding of the cell of origin of osteosarcoma as well as the precise roles of cytokines and cells of the immune system on cells of osteoid lineage will be required to successfully treat metastatic osteosarcomas. Once a more complete understanding of the effects on the immune system of common chemotherapeutics against osteosarcoma is achieved, a multimodal approach consisting of chemotherapy and surgery together with (targeted)

immunotherapy might tip the balance towards a long lasting and specific anti-cancer response.

9 References

1. Mirabello L, Troisi RJ, Savage SA. Osteosarcoma incidence and survival rates from 1973 to 2004: data from the Surveillance, Epidemiology, and End Results Program. Cancer. 2009;115:1531-43.
2. Christine Bouchardy J-ML, Claudia Kühni. Krebs in der Schweiz - Stand und Entwicklung von 1983 bis 2007. Statistik der Schweiz. Neuchâtel: Bundesamt für Statistik (BFS), National Institute for Cancer Epidemiology and Registration (NICER), Schweizer Kinderkrebsregister (SKKR); 2011.
3. Howlader N NA, Krapcho M, Garshell J, Neyman N, Altekruse SF, Kosary CL, Yu M, Ruhl J, Tatalovich Z, Cho H, Mariotto A, Lewis DR, Chen HS, Feuer EJ, Cronin KA. SEER Cancer Statistics Review, 1975-2010. November 2012 ed: National Cancer Institute. Bethesda, MD; 2012.
4. Allison DC, Carney SC, Ahlmann ER, Hendifar A, Chawla S, Fedenko A, et al. A meta-analysis of osteosarcoma outcomes in the modern medical era. Sarcoma. 2012;2012:704872.
5. Cotterill SJ, Wright CM, Pearce MS, Craft AW. Stature of young people with malignant bone tumors. Pediatr Blood Cancer. 2004;42:59-63.
6. Seton M. Paget disease of bone: diagnosis and drug therapy. Cleve Clin J Med. 2013;80:452-62.
7. Hansen MF, Seton M, Merchant A. Osteosarcoma in Paget's disease of bone. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research. 2006;21 Suppl 2:P58-63.
8. Savage SA, Mirabello L. Using epidemiology and genomics to understand osteosarcoma etiology. Sarcoma. 2011;2011:548151.
9. Longhi A, Errani C, De Paolis M, Mercuri M, Bacci G. Primary bone osteosarcoma in the pediatric age: state of the art. Cancer treatment reviews. 2006;32:423-36.
10. Bacci G, Mercuri M, Longhi A, Ferrari S, Bertoni F, Versari M, et al. Grade of chemotherapy-induced necrosis as a predictor of local and systemic control in 881 patients with non-metastatic osteosarcoma of the extremities treated with neoadjuvant chemotherapy in a single institution. European journal of cancer. 2005;41:2079-85.
11. Ragland BD, Bell WC, Lopez RR, Siegal GP. Cytogenetics and molecular biology of osteosarcoma. Lab Invest. 2002;82:365-73.
12. Marina N, Gebhardt M, Teot L, Gorlick R. Biology and therapeutic advances for pediatric osteosarcoma. Oncologist. 2004;9:422-41.
13. Ritter J, Bielack SS. Osteosarcoma. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO. 2010;21 Suppl 7:vii320-5.
14. Aisen AM, Martel W, Braunstein EM, McMillin KI, Phillips WA, Kling TF. MRI and CT evaluation of primary bone and soft-tissue tumors. AJR Am J Roentgenol. 1986;146:749-56.
15. Donohoe KJ, Henkin RE, Royal HD, Brown ML, Collier BD, O'Mara RE, et al. Procedure guideline for bone scintigraphy: 1.0. Society of Nuclear Medicine. Journal of nuclear medicine : official publication, Society of Nuclear Medicine. 1996;37:1903-6.
16. Rybak LD, Rosenthal DI. Radiological imaging for the diagnosis of bone metastases. Q J Nucl Med. 2001;45:53-64.

17. Byun BH, Kong CB, Lim I, Kim BI, Choi CW, Song WS, et al. Comparison of (18)F-FDG PET/CT and (99 m)Tc-MDP bone scintigraphy for detection of bone metastasis in osteosarcoma. Skeletal Radiol. 2013;42:1673-81.
18. Liu F, Zhang Q, Zhu D, Li Z, Li J, Wang B, et al. Performance of Positron Emission Tomography and Positron Emission Tomography/Computed Tomography Using Fluorine-18-Fluorodeoxyglucose for the Diagnosis, Staging, and Recurrence Assessment of Bone Sarcoma: A Systematic Review and Meta-Analysis. Medicine (Baltimore). 2015;94:e1462.
19. Kager L, Zoubek A, Potschger U, Kastner U, Flege S, Kempf-Bielack B, et al. Primary metastatic osteosarcoma: presentation and outcome of patients treated on neoadjuvant Cooperative Osteosarcoma Study Group protocols. J Clin Oncol. 2003;21:2011-8.
20. Bone sarcomas: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO. 2014;25 Suppl 3:iii113-23.
21. Bertoni F, Bacchini P, Staals EL, Davidovitz P. Dedifferentiated parosteal osteosarcoma: the experience of the Rizzoli Institute. Cancer. 2005;103:2373-82.
22. D'Andrea K, Dreyer J, Fahim DK. Utility of Preoperative Magnetic Resonance Imaging Coregistered with Intraoperative Computed Tomographic Scan for the Resection of Complex Tumors of the Spine. World Neurosurg. 2015;84:1804-15.
23. Yedururi S, Morani AC, Gladish GW, Vallabhaneni S, Anderson PM, Hughes D, et al. Cardiovascular involvement by osteosarcoma: an analysis of 20 patients. Pediatr Radiol. 2016;46:21-33.
24. Enneking WF, Spanier SS, Goodman MA. A system for the surgical staging of musculoskeletal sarcoma. Clinical orthopaedics and related research. 1980:106-20.
25. Stacy GS, Mahal RS, Peabody TD. Staging of bone tumors: a review with illustrative examples. AJR Am J Roentgenol. 2006;186:967-76.
26. Vasquez L, Tarrillo F, Oscanoa M, Maza I, Geronimo J, Paredes G, et al. Analysis of Prognostic Factors in High-Grade Osteosarcoma of the Extremities in Children: A 15-Year Single-Institution Experience. Front Oncol. 2016;6:22.
27. Nataraj V, Rastogi S, Khan SA, Sharma MC, Agarwala S, Vishnubhatla S, et al. Prognosticating metastatic osteosarcoma treated with uniform chemotherapy protocol without high dose methotrexate and delayed metastasectomy: a single center experience of 102 patients. Clin Transl Oncol. 2016.
28. Rasalkar DD, Chu WC, Lee V, Paunipagar BK, Cheng FW, Li CK. Pulmonary metastases in children with osteosarcoma: characteristics and impact on patient survival. Pediatr Radiol. 2011;41:227-36.
29. Link MP, Goorin AM, Miser AW, Green AA, Pratt CB, Belasco JB, et al. The effect of adjuvant chemotherapy on relapse-free survival in patients with osteosarcoma of the extremity. N Engl J Med. 1986;314:1600-6.
30. Hauben EI, Weeden S, Pringle J, Van Marck EA, Hogendoorn PC. Does the histological subtype of high-grade central osteosarcoma influence the response to treatment with chemotherapy and does it affect overall survival? A study on 570 patients of two consecutive trials of the European Osteosarcoma Intergroup. European journal of cancer. 2002;38:1218-25.
31. Bielack SS, Kempf-Bielack B, Delling G, Exner GU, Flege S, Helmke K, et al. Prognostic factors in high-grade osteosarcoma of the extremities or trunk: an analysis of 1,702 patients treated on neoadjuvant cooperative osteosarcoma study group protocols. J Clin Oncol. 2002;20:776-90.
32. Ferrari S, Bertoni F, Mercuri M, Picci P, Giacomini S, Longhi A, et al. Predictive factors of disease-free survival for non-metastatic osteosarcoma of the

extremity: an analysis of 300 patients treated at the Rizzoli Institute. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO. 2001;12:1145-50.
33. Yong B, Tan P, Yin J, Zou C, Xie X, Wang J, et al. Suboptimal chemotherapy is an adverse prognostic factor in osteosarcoma. World J Surg Oncol. 2012;10:191.
34. Jeys LM, Grimer RJ, Carter SR, Tillman RM, Abudu A. Post operative infection and increased survival in osteosarcoma patients: are they associated? Ann Surg Oncol. 2007;14:2887-95.
35. McTiernan A, Jinks RC, Sydes MR, Uscinska B, Hook JM, van Glabbeke M, et al. Presence of chemotherapy-induced toxicity predicts improved survival in patients with localised extremity osteosarcoma treated with doxorubicin and cisplatin: a report from the European Osteosarcoma Intergroup. Eur J Cancer. 2012;48:703-12.
36. Chen YU, Xu SF, Xu M, Yu XC. Postoperative infection and survival in osteosarcoma patients: Reconsideration of immunotherapy for osteosarcoma. Mol Clin Oncol. 2015;3:495-500.
37. Moore C, Eslin D, Levy A, Roberson J, Giusti V, Sutphin R. Prognostic significance of early lymphocyte recovery in pediatric osteosarcoma. Pediatr Blood Cancer. 2010;55:1096-102.
38. Abarrategi A, Tornin J, Martinez-Cruzado L, Hamilton A, Martinez-Campos E, Rodrigo JP, et al. Osteosarcoma: Cells-of-Origin, Cancer Stem Cells, and Targeted Therapies. Stem Cells Int. 2016;2016:3631764.
39. Mutsaers AJ, Walkley CR. Cells of origin in osteosarcoma: mesenchymal stem cells or osteoblast committed cells? Bone. 2014;62:56-63.
40. Rubio R, Abarrategi A, Garcia-Castro J, Martinez-Cruzado L, Suarez C, Tornin J, et al. Bone environment is essential for osteosarcoma development from transformed mesenchymal stem cells. Stem Cells. 2014;32:1136-48.
41. Kuijjer ML, Hogendoorn PC, Cleton-Jansen AM. Genome-wide analyses on high-grade osteosarcoma: making sense of a genomically most unstable tumor. International journal of cancer. 2013;133:2512-21.
42. Wittrant Y, Theoleyre S, Chipoy C, Padrines M, Blanchard F, Heymann D, et al. RANKL/RANK/OPG: new therapeutic targets in bone tumours and associated osteolysis. Biochimica et biophysica acta. 2004;1704:49-57.
43. Orr FW, Lee J, Duivenvoorden WC, Singh G. Pathophysiologic interactions in skeletal metastasis. Cancer. 2000;88:2912-8.
44. Guise TA, Chirgwin JM. Transforming growth factor-beta in osteolytic breast cancer bone metastases. Clinical orthopaedics and related research. 2003:S32-8.
45. Hauschka PV, Mavrakos AE, Iafrati MD, Doleman SE, Klagsbrun M. Growth factors in bone matrix. Isolation of multiple types by affinity chromatography on heparin-Sepharose. J Biol Chem. 1986;261:12665-74.
46. Lamora A, Talbot J, Bougras G, Amiaud J, Leduc M, Chesneau J, et al. Overexpression of smad7 blocks primary tumor growth and lung metastasis development in osteosarcoma. Clinical cancer research : an official journal of the American Association for Cancer Research. 2014;20:5097-112.
47. Bridge JA, Nelson M, McComb E, McGuire MH, Rosenthal H, Vergara G, et al. Cytogenetic findings in 73 osteosarcoma specimens and a review of the literature. Cancer Genet Cytogenet. 1997;95:74-87.
48. Tarkkanen M, Karhu R, Kallioniemi A, Elomaa I, Kivioja AH, Nevalainen J, et al. Gains and losses of DNA sequences in osteosarcomas by comparative genomic hybridization. Cancer research. 1995;55:1334-8.
49. Egas-Bejar D, Anderson PM, Agarwal R, Corrales-Medina F, Devarajan E, Huh WW, et al. Theranostic Profiling for Actionable Aberrations in Advanced High

Risk Osteosarcoma with Aggressive Biology Reveals High Molecular Diversity: The Human Fingerprint Hypothesis. Oncoscience. 2014;1:167-79.
50. Chen X, Bahrami A, Pappo A, Easton J, Dalton J, Hedlund E, et al. Recurrent somatic structural variations contribute to tumorigenesis in pediatric osteosarcoma. Cell Rep. 2014;7:104-12.
51. Perry JA, Kiezun A, Tonzi P, Van Allen EM, Carter SL, Baca SC, et al. Complementary genomic approaches highlight the PI3K/mTOR pathway as a common vulnerability in osteosarcoma. Proceedings of the National Academy of Sciences of the United States of America. 2014;111:E5564-73.
52. Jones KB. Transposon mutagenesis disentangles osteosarcoma genetic drivers. Nat Genet. 2015;47:564-5.
53. May WA, Lessnick SL, Braun BS, Klemsz M, Lewis BC, Lunsford LB, et al. The Ewing's sarcoma EWS/FLI-1 fusion gene encodes a more potent transcriptional activator and is a more powerful transforming gene than FLI-1. Mol Cell Biol. 1993;13:7393-8.
54. Kuijjer ML, Rydbeck H, Kresse SH, Buddingh EP, Lid AB, Roelofs H, et al. Identification of osteosarcoma driver genes by integrative analysis of copy number and gene expression data. Genes Chromosomes Cancer. 2012;51:696-706.
55. Stephens PJ, Greenman CD, Fu B, Yang F, Bignell GR, Mudie LJ, et al. Massive genomic rearrangement acquired in a single catastrophic event during cancer development. Cell. 2011;144:27-40.
56. Smida J, Baumhoer D, Rosemann M, Walch A, Bielack S, Poremba C, et al. Genomic alterations and allelic imbalances are strong prognostic predictors in osteosarcoma. Clinical cancer research : an official journal of the American Association for Cancer Research. 2010;16:4256-67.
57. Kuijjer ML, Namlos HM, Hauben EI, Machado I, Kresse SH, Serra M, et al. mRNA expression profiles of primary high-grade central osteosarcoma are preserved in cell lines and xenografts. BMC Med Genomics. 2011;4:66.
58. Wadayama B, Toguchida J, Shimizu T, Ishizaki K, Sasaki MS, Kotoura Y, et al. Mutation spectrum of the retinoblastoma gene in osteosarcomas. Cancer research. 1994;54:3042-8.
59. Tsuchiya T, Sekine K, Hinohara S, Namiki T, Nobori T, Kaneko Y. Analysis of the p16INK4, p14ARF, p15, TP53, and MDM2 genes and their prognostic implications in osteosarcoma and Ewing sarcoma. Cancer Genet Cytogenet. 2000;120:91-8.
60. Mohseny AB, Tieken C, van der Velden PA, Szuhai K, de Andrea C, Hogendoorn PC, et al. Small deletions but not methylation underlie CDKN2A/p16 loss of expression in conventional osteosarcoma. Genes Chromosomes Cancer. 2010;49:1095-103.
61. Freeman SS, Allen SW, Ganti R, Wu J, Ma J, Su X, et al. Copy number gains in EGFR and copy number losses in PTEN are common events in osteosarcoma tumors. Cancer. 2008;113:1453-61.
62. Duhamel LA, Ye H, Halai D, Idowu BD, Presneau N, Tirabosco R, et al. Frequency of Mouse Double Minute 2 (MDM2) and Mouse Double Minute 4 (MDM4) amplification in parosteal and conventional osteosarcoma subtypes. Histopathology. 2012;60:357-9.
63. Yang J, Yang D, Sun Y, Sun B, Wang G, Trent JC, et al. Genetic amplification of the vascular endothelial growth factor (VEGF) pathway genes, including VEGFA, in human osteosarcoma. Cancer. 2011;117:4925-38.

64. Pompetti F, Rizzo P, Simon RM, Freidlin B, Mew DJ, Pass HI, et al. Oncogene alterations in primary, recurrent, and metastatic human bone tumors. J Cell Biochem. 1996;63:37-50.
65. Papachristou DJ, Batistatou A, Sykiotis GP, Varakis I, Papavassiliou AG. Activation of the JNK-AP-1 signal transduction pathway is associated with pathogenesis and progression of human osteosarcomas. Bone. 2003;32:364-71.
66. Weekes D, Kashima TG, Zandueta C, Perurena N, Thomas DP, Sunters A, et al. Regulation of osteosarcoma cell lung metastasis by the c-Fos/AP-1 target FGFR1. Oncogene. 2015.
67. Ulaner GA, Vu TH, Li T, Hu JF, Yao XM, Yang Y, et al. Loss of imprinting of IGF2 and H19 in osteosarcoma is accompanied by reciprocal methylation changes of a CTCF-binding site. Hum Mol Genet. 2003;12:535-49.
68. Zhang XH, Jin X, Malladi S, Zou Y, Wen YH, Brogi E, et al. Selection of bone metastasis seeds by mesenchymal signals in the primary tumor stroma. Cell. 2013;154:1060-73.
69. Kaji H, Sugimoto T, Kanatani M, Nishiyama K, Nasu M, Chihara K. Insulin-like growth factor-I mediates osteoclast-like cell formation stimulated by parathyroid hormone. J Cell Physiol. 1997;172:55-62.
70. Sadikovic B, Yoshimoto M, Chilton-MacNeill S, Thorner P, Squire JA, Zielenska M. Identification of interactive networks of gene expression associated with osteosarcoma oncogenesis by integrated molecular profiling. Hum Mol Genet. 2009;18:1962-75.
71. Khanna C, Wan X, Bose S, Cassaday R, Olomu O, Mendoza A, et al. The membrane-cytoskeleton linker ezrin is necessary for osteosarcoma metastasis. Nat Med. 2004;10:182-6.
72. Bretscher A, Edwards K, Fehon RG. ERM proteins and merlin: integrators at the cell cortex. Nat Rev Mol Cell Biol. 2002;3:586-99.
73. Perissinotto E, Cavalloni G, Leone F, Fonsato V, Mitola S, Grignani G, et al. Involvement of chemokine receptor 4/stromal cell-derived factor 1 system during osteosarcoma tumor progression. Clinical cancer research : an official journal of the American Association for Cancer Research. 2005;11:490-7.
74. Laverdiere C, Hoang BH, Yang R, Sowers R, Qin J, Meyers PA, et al. Messenger RNA expression levels of CXCR4 correlate with metastatic behavior and outcome in patients with osteosarcoma. Clinical cancer research : an official journal of the American Association for Cancer Research. 2005;11:2561-7.
75. Sanchez-Diaz PC, Hsiao TH, Zou Y, Sugalski AJ, Heim-Hall J, Chen Y, et al. In silico functional analyses and discovery of survival-associated microRNA signatures in pediatric osteosarcoma. Oncoscience. 2014;1:599-608.
76. Sampson VB, Yoo S, Kumar A, Vetter NS, Kolb EA. MicroRNAs and Potential Targets in Osteosarcoma: Review. Front Pediatr. 2015;3:69.
77. Moriarity BS, Otto GM, Rahrmann EP, Rathe SK, Wolf NK, Weg MT, et al. A Sleeping Beauty forward genetic screen identifies new genes and pathways driving osteosarcoma development and metastasis. Nat Genet. 2015;47:615-24.
78. Valastyan S, Weinberg RA. Tumor metastasis: molecular insights and evolving paradigms. Cell. 2011;147:275-92.
79. Luzzi KJ, MacDonald IC, Schmidt EE, Kerkvliet N, Morris VL, Chambers AF, et al. Multistep nature of metastatic inefficiency: dormancy of solitary cells after successful extravasation and limited survival of early micrometastases. Am J Pathol. 1998;153:865-73.

80. Wagenblast E, Soto M, Gutierrez-Angel S, Hartl CA, Gable AL, Maceli AR, et al. A model of breast cancer heterogeneity reveals vascular mimicry as a driver of metastasis. Nature. 2015;520:358-62.
81. Kim MY, Oskarsson T, Acharyya S, Nguyen DX, Zhang XH, Norton L, et al. Tumor self-seeding by circulating cancer cells. Cell. 2009;139:1315-26.
82. Hattinger CM, Pasello M, Ferrari S, Picci P, Serra M. Emerging drugs for high-grade osteosarcoma. Expert Opin Emerg Drugs. 2010;15:615-34.
83. Pakos EE, Nearchou AD, Grimer RJ, Koumoullis HD, Abudu A, Bramer JA, et al. Prognostic factors and outcomes for osteosarcoma: an international collaboration. European journal of cancer. 2009;45:2367-75.
84. Osborne TS, Khanna C. A review of the association between osteosarcoma metastasis and protein translation. J Comp Pathol. 2012;146:132-42.
85. Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Matera J, Miller MC, et al. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. N Engl J Med. 2004;351:781-91.
86. Schleiermacher G, Peter M, Oberlin O, Philip T, Rubie H, Mechinaud F, et al. Increased risk of systemic relapses associated with bone marrow micrometastasis and circulating tumor cells in localized ewing tumor. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2003;21:85-91.
87. Wong IH, Chan AT, Johnson PJ. Quantitative analysis of circulating tumor cells in peripheral blood of osteosarcoma patients using osteoblast-specific messenger RNA markers: a pilot study. Clinical cancer research : an official journal of the American Association for Cancer Research. 2000;6:2183-8.
88. Zhang Y, Ma Q, Liu T, Ke S, Jiang K, Wen Y, et al. Tumor self-seeding by circulating tumor cells in nude mouse models of human osteosarcoma and a preliminary study of its mechanisms. Journal of cancer research and clinical oncology. 2014;140:329-40.
89. Kempf-Bielack B, Bielack SS, Jurgens H, Branscheid D, Berdel WE, Exner GU, et al. Osteosarcoma relapse after combined modality therapy: an analysis of unselected patients in the Cooperative Osteosarcoma Study Group (COSS). Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2005;23:559-68.
90. PosthumaDeBoer J, Witlox MA, Kaspers GJ, van Royen BJ. Molecular alterations as target for therapy in metastatic osteosarcoma: a review of literature. Clin Exp Metastasis. 2011;28:493-503.
91. Krishnan K, Khanna C, Helman LJ. The biology of metastases in pediatric sarcomas. Cancer J. 2005;11:306-13.
92. Paget S. The distribution of secondary growths in cancer of the breast. The Lancet. 1889;133:571-3.
93. Hoshino A, Costa-Silva B, Shen TL, Rodrigues G, Hashimoto A, Tesic Mark M, et al. Tumour exosome integrins determine organotropic metastasis. Nature. 2015;527:329-35.
94. Endo-Munoz L, Cai N, Cumming A, Macklin R, Merida de Long L, Topkas E, et al. Progression of Osteosarcoma from a Non-Metastatic to a Metastatic Phenotype Is Causally Associated with Activation of an Autocrine and Paracrine uPA Axis. PloS one. 2015;10:e0133592.
95. Qian BZ, Pollard JW. Macrophage diversity enhances tumor progression and metastasis. Cell. 2010;141:39-51.
96. Buddingh EP, Kuijjer ML, Duim RA, Burger H, Agelopoulos K, Myklebost O, et al. Tumor-infiltrating macrophages are associated with metastasis suppression in high-grade osteosarcoma: a rationale for treatment with macrophage activating

agents. Clinical cancer research : an official journal of the American Association for Cancer Research. 2011;17:2110-9.
97. Pahl JH, Kwappenberg KM, Varypataki EM, Santos SJ, Kuijjer ML, Mohamed S, et al. Macrophages inhibit human osteosarcoma cell growth after activation with the bacterial cell wall derivative liposomal muramyl tripeptide in combination with interferon-gamma. J Exp Clin Cancer Res. 2014;33:27.
98. Odagiri H, Kadomatsu T, Endo M, Masuda T, Morioka MS, Fukuhara S, et al. The secreted protein ANGPTL2 promotes metastasis of osteosarcoma cells through integrin alpha5beta1, p38 MAPK, and matrix metalloproteinases. Sci Signal. 2014;7:ra7.
99. Vermeer PD, Denker J, Estin M, Moninger TO, Keshavjee S, Karp P, et al. MMP9 modulates tight junction integrity and cell viability in human airway epithelia. Am J Physiol Lung Cell Mol Physiol. 2009;296:L751-62.
100. Kansara M, Teng MW, Smyth MJ, Thomas DM. Translational biology of osteosarcoma. Nat Rev Cancer. 2014;14:722-35.
101. Picci P. Osteosarcoma (osteogenic sarcoma). Orphanet J Rare Dis. 2007;2:6.
102. Goorin AM, Harris MB, Bernstein M, Ferguson W, Devidas M, Siegal GP, et al. Phase II/III trial of etoposide and high-dose ifosfamide in newly diagnosed metastatic osteosarcoma: a pediatric oncology group trial. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2002;20:426-33.
103. Bacci G, Ferrari S, Tienghi A, Bertoni F, Mercuri M, Longhi A, et al. A comparison of methods of loco-regional chemotherapy combined with systemic chemotherapy as neo-adjuvant treatment of osteosarcoma of the extremity. Eur J Surg Oncol. 2001;27:98-104.
104. Goorin AM, Schwartzentruber DJ, Devidas M, Gebhardt MC, Ayala AG, Harris MB, et al. Presurgical chemotherapy compared with immediate surgery and adjuvant chemotherapy for nonmetastatic osteosarcoma: Pediatric Oncology Group Study POG-8651. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2003;21:1574-80.
105. Salzer-Kuntschik M, Brand G, Delling G. [Determination of the degree of morphological regression following chemotherapy in malignant bone tumors]. Pathologe. 1983;4:135-41.
106. Grimer RJ. Surgical options for children with osteosarcoma. The Lancet Oncology. 2005;6:85-92.
107. Bertrand TE, Cruz A, Binitie O, Cheong D, Letson GD. Do Surgical Margins Affect Local Recurrence and Survival in Extremity, Nonmetastatic, High-grade Osteosarcoma? Clinical orthopaedics and related research. 2016;474:677-83.
108. Bacci G, Picci P, Ruggieri P, Mercuri M, Avella M, Capanna R, et al. Primary chemotherapy and delayed surgery (neoadjuvant chemotherapy) for osteosarcoma of the extremities. The Istituto Rizzoli Experience in 127 patients treated preoperatively with intravenous methotrexate (high versus moderate doses) and intraarterial cisplatin. Cancer. 1990;65:2539-53.
109. Marina N, Bielack S, Whelan J, Smeland S, Krailo M, Sydes MR, et al. International collaboration is feasible in trials for rare conditions: the EURAMOS experience. Cancer treatment and research. 2009;152:339-53.
110. Medical Research Council CTU. EURAMOS I : Trial Design. 2006 [cited 2016 4.4.2016]; Available from: http://www.ctu.mrc.ac.uk/euramos/e_i_design.asp
111. Whelan JS, Bielack SS, Marina N, Smeland S, Jovic G, Hook JM, et al. EURAMOS-1, an international randomised study for osteosarcoma: results from pre-randomisation treatment. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO. 2015;26:407-14.

112. Jia SF, An T, Worth L, Kleinerman ES. Interferon-alpha enhances the sensitivity of human osteosarcoma cells to etoposide. J Interferon Cytokine Res. 1999;19:617-24.
113. Masuda S, Fukuma H, Beppu Y. Antitumor effect of human leukocyte interferon on human osteosarcoma transplanted into nude mice. European journal of cancer & clinical oncology. 1983;19:1521-8.
114. Brosjo O, Bauer HC, Brostrom LA, Nilsonne U, Nilsson OS, Reinholt FP, et al. Influence of human alpha-interferon on four human osteosarcoma xenografts in nude mice. Cancer research. 1985;45:5598-602.
115. Forster S, Triffitt JT, Bauer HC, Brosjo O, Nilsson OS, Smith R, et al. Interferon-inhibited human osteosarcoma xenografts induce host bone in nude mice. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research. 1988;3:199-202.
116. Muller CR, Smeland S, Bauer HC, Saeter G, Strander H. Interferon-alpha as the only adjuvant treatment in high-grade osteosarcoma: long term results of the Karolinska Hospital series. Acta Oncol. 2005;44:475-80.
117. Winkler K, Beron G, Kotz R, Salzer-Kuntschik M, Beck J, Beck W, et al. Neoadjuvant chemotherapy for osteogenic sarcoma: results of a Cooperative German/Austrian study. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 1984;2:617-24.
118. Bielack SS, Smeland S, Whelan JS, Marina N, Jovic G, Hook JM, et al. Methotrexate, Doxorubicin, and Cisplatin (MAP) Plus Maintenance Pegylated Interferon Alfa-2b Versus MAP Alone in Patients With Resectable High-Grade Osteosarcoma and Good Histologic Response to Preoperative MAP: First Results of the EURAMOS-1 Good Response Randomized Controlled Trial. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2015;33:2279-87.
119. Takayanagi H, Kim S, Matsuo K, Suzuki H, Suzuki T, Sato K, et al. RANKL maintains bone homeostasis through c-Fos-dependent induction of interferon-beta. Nature. 2002;416:744-9.
120. el-Khateeb M, Appleton TG, Gahan LR, Charles BG, Berners-Price SJ, Bolton AM. Reactions of cisplatin hydrolytes with methionine, cysteine, and plasma ultrafiltrate studied by a combination of HPLC and NMR techniques. J Inorg Biochem. 1999;77:13-21.
121. Kelland L. The resurgence of platinum-based cancer chemotherapy. Nat Rev Cancer. 2007;7:573-84.
122. Nitiss JL. Targeting DNA topoisomerase II in cancer chemotherapy. Nat Rev Cancer. 2009;9:338-50.
123. Kerbusch T, de Kraker J, Keizer HJ, van Putten JW, Groen HJ, Jansen RL, et al. Clinical pharmacokinetics and pharmacodynamics of ifosfamide and its metabolites. Clin Pharmacokinet. 2001;40:41-62.
124. Dong Q, Barsky D, Colvin ME, Melius CF, Ludeman SM, Moravek JF, et al. A structural basis for a phosphoramide mustard-induced DNA interstrand cross-link at 5'-d(GAC). Proceedings of the National Academy of Sciences of the United States of America. 1995;92:12170-4.
125. Hess JA, Khasawneh MK. Cancer metabolism and oxidative stress: Insights into carcinogenesis and chemotherapy via the non-dihydrofolate reductase effects of methotrexate. BBA Clin. 2015;3:152-61.
126. Assi H, Missenard G, Terrier P, Le Pechoux C, Bonvalot S, Vanel D, et al. Intensive induction chemotherapy without methotrexate in adult patients with

localized osteosarcoma: results of the Institut Gustave-Roussy phase II trial. Curr Oncol. 2010;17:23-31.
127. Ferrari S, Smeland S, Mercuri M, Bertoni F, Longhi A, Ruggieri P, et al. Neoadjuvant chemotherapy with high-dose Ifosfamide, high-dose methotrexate, cisplatin, and doxorubicin for patients with localized osteosarcoma of the extremity: a joint study by the Italian and Scandinavian Sarcoma Groups. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2005;23:8845-52.
128. Bacci G, Ferrari S, Bertoni F, Ruggieri P, Picci P, Longhi A, et al. Long-term outcome for patients with nonmetastatic osteosarcoma of the extremity treated at the istituto ortopedico rizzoli according to the istituto ortopedico rizzoli/osteosarcoma-2 protocol: an updated report. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2000;18:4016-27.
129. Pratt CB, Meyer WH, Luo X, Cain AM, Kaste SC, Pappo AS, et al. Second malignant neoplasms occuring in survivors of osteosarcoma. Cancer. 1997;80:960-5.
130. Kim SH, Shin KH, Seok SO, Cho YJ, Noh JK, Suh JS, et al. Secondary malignant neoplasms after osteosarcoma: early onset and cumulative alkylating agent dose dependency. Ann Surg Oncol. 2015;22:859-65.
131. Hagleitner MM, de Bont ES, Te Loo DM. Survival trends and long-term toxicity in pediatric patients with osteosarcoma. Sarcoma. 2012;2012:636405.
132. Janeway KA, Grier HE. Sequelae of osteosarcoma medical therapy: a review of rare acute toxicities and late effects. The Lancet Oncology. 2010;11:670-8.
133. Meyers PA, Schwartz CL, Krailo M, Kleinerman ES, Betcher D, Bernstein ML, et al. Osteosarcoma: a randomized, prospective trial of the addition of ifosfamide and/or muramyl tripeptide to cisplatin, doxorubicin, and high-dose methotrexate. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2005;23:2004-11.
134. Fuchs N, Bielack SS, Epler D, Bieling P, Delling G, Korholz D, et al. Long-term results of the co-operative German-Austrian-Swiss osteosarcoma study group's protocol COSS-86 of intensive multidrug chemotherapy and surgery for osteosarcoma of the limbs. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO. 1998;9:893-9.
135. Pabla N, Dong Z. Cisplatin nephrotoxicity: mechanisms and renoprotective strategies. Kidney Int. 2008;73:994-1007.
136. Widemann BC, Balis FM, Kempf-Bielack B, Bielack S, Pratt CB, Ferrari S, et al. High-dose methotrexate-induced nephrotoxicity in patients with osteosarcoma. Cancer. 2004;100:2222-32.
137. Klopp CT, Alford TC, Bateman J, Berry GN, Winship T. Fractionated intra-arterial cancer; chemotherapy with methyl bis amine hydrochloride; a preliminary report. Ann Surg. 1950;132:811-32.
138. Chuang VP, Wallace S, Benjamin RS, Jaffe N, Ayala A, Murray J, et al. The therapy of osteosarcoma by intraarterial cis-platinum an limb preservation. Cardiovasc Intervent Radiol. 1981;4:229-35.
139. Jaffe N, Knapp J, Chuang VP, Wallace S, Ayala A, Murray J, et al. Osteosarcoma: intra-arterial treatment of the primary tumor with cis-diammine-dichloroplatinum II (CDP). Angiographic, pathologic, and pharmacologic studies. Cancer. 1983;51:402-7.
140. Mavligit GM, Benjamin R, Patt YZ, Jaffe N, Chuang V, Wallace S, et al. Intraarterial cis-platinum for patients with inoperable skeletal tumors. Cancer. 1981;48:1-4.

141. Stewart DJ, Benjamin RS, Zimmerman S, Caprioli RM, Wallace S, Chuang V, et al. Clinical pharmacology of intraarterial cis-diamminedichloroplatinum(II). Cancer Res. 1983;43:917-20.
142. Jaffe N, Raymond AK, Ayala A, Carrasco CH, Wallace S, Robertson R, et al. Effect of cumulative courses of intraarterial cis-diamminedichloroplatin-II on the primary tumor in osteosarcoma. Cancer. 1989;63:63-7.
143. Wilkins RM, Cullen JW, Camozzi AB, Jamroz BA, Odom L. Improved survival in primary nonmetastatic pediatric osteosarcoma of the extremity. Clin Orthop Relat Res. 2005;438:128-36.
144. Hong S, Shin SJ, Jung M, Jeong J, Lee YJ, Shin KH, et al. Comparison of long-term outcome between doublet and triplet neoadjuvant chemotherapy in non-metastatic osteosarcoma of the extremity. Oncology. 2011;80:107-17.
145. Stephens FO, Tattersall MH, Marsden W, Waugh RC, Green D, McCarthy SW. Regional chemotherapy with the use of cisplatin and doxorubicin as primary treatment for advanced sarcomas in shoulder, pelvis, and thigh. Cancer. 1987;60:724-35.
146. Bramwell VH, Burgers M, Sneath R, Souhami R, van Oosterom AT, Voute PA, et al. A comparison of two short intensive adjuvant chemotherapy regimens in operable osteosarcoma of limbs in children and young adults: the first study of the European Osteosarcoma Intergroup. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 1992;10:1579-91.
147. Bielack S, Kempf-Bielack B, Schwenzer D, Birkfellner T, Delling G, Ewerbeck V, et al. [Neoadjuvant therapy for localized osteosarcoma of extremities. Results from the Cooperative osteosarcoma study group COSS of 925 patients]. Klinische Padiatrie. 1999;211:260-70.
148. Ferrari S, Mercuri M, Picci P, Bertoni F, Brach del Prever A, Tienghi A, et al. Nonmetastatic osteosarcoma of the extremity: results of a neoadjuvant chemotherapy protocol (IOR/OS-3) with high-dose methotrexate, intraarterial or intravenous cisplatin, doxorubicin, and salvage chemotherapy based on histologic tumor response. Tumori. 1999;85:458-64.
149. Hugate RR, Wilkins RM, Kelly CM, Madsen W, Hinshaw I, Camozzi AB. Intraarterial chemotherapy for extremity osteosarcoma and MFH in adults. Clin Orthop Relat Res. 2008;466:1292-301.
150. Tunn PU, Schmidt-Peter P, Pomraenke D, Hohenberger P. Osteosarcoma in children: long-term functional analysis. Clinical orthopaedics and related research. 2004:212-7.
151. Winkler K, Bielack S, Delling G, Salzer-Kuntschik M, Kotz R, Greenshaw C, et al. Effect of intraarterial versus intravenous cisplatin in addition to systemic doxorubicin, high-dose methotrexate, and ifosfamide on histologic tumor response in osteosarcoma (study COSS-86). Cancer. 1990;66:1703-10.
152. Rasch CR, Hauptmann M, Schornagel J, Wijers O, Buter J, Gregor T, et al. Intra-arterial versus intravenous chemoradiation for advanced head and neck cancer: Results of a randomized phase 3 trial. Cancer. 2010;116:2159-65.
153. Suzuki S, Yasunaga H, Matsui H, Fushimi K, Saito Y, Yamasoba T. Cerebral infarction after intraarterial and intravenous chemoradiotherapy for head and neck cancer: A retrospective analysis using a Japanese inpatient database. Head & neck. 2016.
154. Imbesi F, Marchioni E, Benericetti E, Zappoli F, Galli A, Corato M, et al. A randomized phase III study: comparison between intravenous and intraarterial ACNU administration in newly diagnosed primary glioblastomas. Anticancer research. 2006;26:553-8.

155. Stewart DJ, Grahovac Z, Hugenholtz H, DaSilva V, Richard MT, Benoit B, et al. Feasibility study of intraarterial vs intravenous cisplatin, BCNU, and teniposide combined with systemic cisplatin, teniposide, cytosine arabinoside, glycerol and mannitol in the treatment of primary and metastatic brain tumors. J Neurooncol. 1993;17:71-9.
156. Rodriguez CO, Jr. Using canine osteosarcoma as a model to assess efficacy of novel therapies: can old dogs teach us new tricks? Adv Exp Med Biol. 2014;804:237-56.
157. Paoloni M, Davis S, Lana S, Withrow S, Sangiorgi L, Picci P, et al. Canine tumor cross-species genomics uncovers targets linked to osteosarcoma progression. BMC Genomics. 2009;10:625.
158. Powers BE, Withrow SJ, Thrall DE, Straw RC, LaRue SM, Page RL, et al. Percent tumor necrosis as a predictor of treatment response in canine osteosarcoma. Cancer. 1991;67:126-34.
159. Harker G, Stephens F. A report on the comparative response of sheep epidermal squamous-cell carcinoma to intraarterial versus intravenous Cisplatin infusion. Int J Oncol. 1995;7:365-70.
160. Yamada K, Ushio Y, Hayakawa T, Arita N, Huang TY, Nagatani M, et al. Distribution of radiolabeled 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitros ourea hydrochloride in rat brain tumor: intraarterial versus intravenous administration. Cancer research. 1987;47:2123-8.
161. Takeda N, Diksic M, Yamamoto YL. The sequential changes in DNA synthesis, glucose utilization, protein synthesis, and peripheral benzodiazepine receptor density in C6 brain tumors after chemotherapy to predict the response of tumors to chemotherapy. Cancer. 1996;77:1167-79.
162. Schuster JM, Friedman HS, Archer GE, Fuchs HE, McLendon RE, Colvin OM, et al. Intraarterial therapy of human glioma xenografts in athymic rats using 4-hydroperoxycyclophosphamide. Cancer research. 1993;53:2338-43.
163. Wiemann B, Starnes CO. Coley's toxins, tumor necrosis factor and cancer research: a historical perspective. Pharmacol Ther. 1994;64:529-64.
164. Modiano JF, Bellgrau D, Cutter GR, Lana SE, Ehrhart NP, Ehrhart E, et al. Inflammation, apoptosis, and necrosis induced by neoadjuvant fas ligand gene therapy improves survival of dogs with spontaneous bone cancer. Mol Ther. 2012;20:2234-43.
165. Lascelles BD, Dernell WS, Correa MT, Lafferty M, Devitt CM, Kuntz CA, et al. Improved survival associated with postoperative wound infection in dogs treated with limb-salvage surgery for osteosarcoma. Ann Surg Oncol. 2005;12:1073-83.
166. EMA. European public assessment reports. 2009 01/10/2013 [cited 2016 9/5/2016]; Available from: www.ema.europa.eu
167. Ando K, Mori K, Corradini N, Redini F, Heymann D. Mifamurtide for the treatment of nonmetastatic osteosarcoma. Expert Opin Pharmacother. 2011;12:285-92.
168. Kleinerman ES, Jia SF, Griffin J, Seibel NL, Benjamin RS, Jaffe N. Phase II study of liposomal muramyl tripeptide in osteosarcoma: the cytokine cascade and monocyte activation following administration. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 1992;10:1310-6.
169. MacEwen EG, Kurzman ID, Rosenthal RC, Smith BW, Manley PA, Roush JK, et al. Therapy for osteosarcoma in dogs with intravenous injection of liposome-encapsulated muramyl tripeptide. Journal of the National Cancer Institute. 1989;81:935-8.

170. Meyers PA, Schwartz CL, Krailo MD, Healey JH, Bernstein ML, Betcher D, et al. Osteosarcoma: the addition of muramyl tripeptide to chemotherapy improves overall survival--a report from the Children's Oncology Group. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2008;26:633-8.
171. Liu T, Fang XC, Ding Z, Sun ZG, Sun LM, Wang YL. Pre-operative lymphocyte-to-monocyte ratio as a predictor of overall survival in patients suffering from osteosarcoma. FEBS Open Bio. 2015;5:682-7.
172. Fritzsching B, Fellenberg J, Moskovszky L, Sapi Z, Krenacs T, Machado I, et al. CD8/FOXP3-ratio in osteosarcoma microenvironment separates survivors from non-survivors: a multicenter validated retrospective study. Oncoimmunology. 2015;4:e990800.
173. Carswell EA, Old LJ, Kassel RL, Green S, Fiore N, Williamson B. An endotoxin-induced serum factor that causes necrosis of tumors. Proceedings of the National Academy of Sciences of the United States of America. 1975;72:3666-70.
174. Croft M, Duan W, Choi H, Eun SY, Madireddi S, Mehta A. TNF superfamily in inflammatory disease: translating basic insights. Trends Immunol. 2012;33:144-52.
175. Haraoui B, Bykerk V. Etanercept in the treatment of rheumatoid arthritis. Ther Clin Risk Manag. 2007;3:99-105.
176. Peters CP, Eshuis EJ, Toxopeus FM, Hellemons ME, Jansen JM, D'Haens GR, et al. Adalimumab for Crohn's disease: long-term sustained benefit in a population-based cohort of 438 patients. J Crohns Colitis. 2014;8:866-75.
177. Sands BE, Anderson FH, Bernstein CN, Chey WY, Feagan BG, Fedorak RN, et al. Infliximab maintenance therapy for fistulizing Crohn's disease. N Engl J Med. 2004;350:876-85.
178. Zelova H, Hosek J. TNF-alpha signalling and inflammation: interactions between old acquaintances. Inflamm Res. 2013;62:641-51.
179. Bradley JR. TNF-mediated inflammatory disease. The Journal of pathology. 2008;214:149-60.
180. Black RA, Rauch CT, Kozlosky CJ, Peschon JJ, Slack JL, Wolfson MF, et al. A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. Nature. 1997;385:729-33.
181. Grell M, Douni E, Wajant H, Lohden M, Clauss M, Maxeiner B, et al. The transmembrane form of tumor necrosis factor is the prime activating ligand of the 80 kDa tumor necrosis factor receptor. Cell. 1995;83:793-802.
182. Mark KS, Trickler WJ, Miller DW. Tumor necrosis factor-alpha induces cyclooxygenase-2 expression and prostaglandin release in brain microvessel endothelial cells. The Journal of pharmacology and experimental therapeutics. 2001;297:1051-8.
183. Goldblum SE, Sun WL. Tumor necrosis factor-alpha augments pulmonary arterial transendothelial albumin flux in vitro. Am J Physiol. 1990;258:L57-67.
184. Chandrasekharan UM, Siemionow M, Unsal M, Yang L, Poptic E, Bohn J, et al. Tumor necrosis factor alpha (TNF-alpha) receptor-II is required for TNF-alpha-induced leukocyte-endothelial interaction in vivo. Blood. 2007;109:1938-44.
185. Balkwill F, Mantovani A. Inflammation and cancer: back to Virchow? Lancet. 2001;357:539-45.
186. Hardy R, Cooper MS. Bone loss in inflammatory disorders. J Endocrinol. 2009;201:309-20.
187. Moriceau G, Ory B, Gobin B, Verrecchia F, Gouin F, Blanchard F, et al. Therapeutic approach of primary bone tumours by bisphosphonates. Curr Pharm Des. 2010;16:2981-7.

188. Szlosarek P, Charles KA, Balkwill FR. Tumour necrosis factor-alpha as a tumour promoter. European journal of cancer. 2006;42:745-50.
189. Regenass U, Muller M, Curschellas E, Matter A. Anti-tumor effects of tumor necrosis factor in combination with chemotherapeutic agents. International journal of cancer Journal international du cancer. 1987;39:266-73.
190. Ruegg C, Yilmaz A, Bieler G, Bamat J, Chaubert P, Lejeune FJ. Evidence for the involvement of endothelial cell integrin alphaVbeta3 in the disruption of the tumor vasculature induced by TNF and IFN-gamma. Nature medicine. 1998;4:408-14.
191. Havell EA, Fiers W, North RJ. The antitumor function of tumor necrosis factor (TNF), I. Therapeutic action of TNF against an established murine sarcoma is indirect, immunologically dependent, and limited by severe toxicity. J Exp Med. 1988;167:1067-85.
192. Hock H, Dorsch M, Kunzendorf U, Qin Z, Diamantstein T, Blankenstein T. Mechanisms of rejection induced by tumor cell-targeted gene transfer of interleukin 2, interleukin 4, interleukin 7, tumor necrosis factor, or interferon gamma. Proceedings of the National Academy of Sciences of the United States of America. 1993;90:2774-8.
193. Tracey KJ, Cerami A. Tumor necrosis factor: a pleiotropic cytokine and therapeutic target. Annu Rev Med. 1994;45:491-503.
194. Grunhagen DJ, de Wilt JH, Graveland WJ, Verhoef C, van Geel AN, Eggermont AM. Outcome and prognostic factor analysis of 217 consecutive isolated limb perfusions with tumor necrosis factor-alpha and melphalan for limb-threatening soft tissue sarcoma. Cancer. 2006;106:1776-84.
195. Deroose JP, Eggermont AM, van Geel AN, de Wilt JH, Burger JW, Verhoef C. 20 years experience of TNF-based isolated limb perfusion for in-transit melanoma metastases: TNF dose matters. Ann Surg Oncol. 2012;19:627-35.
196. Hernandez J, Cooper J, Babel N, Morton C, Rosemurgy AS. TNFalpha gene delivery therapy for solid tumors. Expert Opin Biol Ther. 2010;10:993-9.
197. Hayashi A, Hayashi H, Chiba T, Sasayama S, Onozaki K. N-acetylneuraminic acid coupled human recombinant TNFalpha exhibits enhanced anti-tumor activity against Meth-A fibrosarcoma and reduced toxicity. Cancer Immunol Immunother. 2007;56:555-62.
198. Borsi L, Balza E, Carnemolla B, Sassi F, Castellani P, Berndt A, et al. Selective targeted delivery of TNFalpha to tumor blood vessels. Blood. 2003;102:4384-92.
199. Eggermont AM, Schraffordt Koops H, Lienard D, Kroon BB, van Geel AN, Hoekstra HJ, et al. Isolated limb perfusion with high-dose tumor necrosis factor-alpha in combination with interferon-gamma and melphalan for nonresectable extremity soft tissue sarcomas: a multicenter trial. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 1996;14:2653-65.
200. Deroose JP, Grunhagen DJ, de Wilt JH, Eggermont AM, Verhoef C. Treatment modifications in tumour necrosis factor-alpha (TNF)-based isolated limb perfusion in patients with advanced extremity soft tissue sarcomas. European journal of cancer. 2015;51:367-73.
201. Van Ginkel RJ, Van Berlo CL, Baas PC, Koops HS, Stuling RV, Elstrodt J, et al. Hyperthermic Isolated Limb Perfusion with TNF alpha and Cisplatin in the Treatment of Osteosarcoma of the Extremities: A Feasibility Study in Healthy Dogs. Sarcoma. 1999;3:89-94.
202. Lejeune FJ, Lienard D, Matter M, Ruegg C. Efficiency of recombinant human TNF in human cancer therapy. Cancer Immun. 2006;6:6.

203. Bickels J, Manusama ER, Gutman M, Eggermont AM, Kollender Y, Abu-Abid S, et al. Isolated limb perfusion with tumour necrosis factor-alpha and melphalan for unresectable bone sarcomas of the lower extremity. Eur J Surg Oncol. 1999;25:509-14.
204. Kato H, Wakabayashi H, Naito Y, Kato S, Nakagawa T, Matsumine A, et al. Anti-tumor necrosis factor therapy inhibits lung metastasis in an osteosarcoma cell line. Oncology. 2015;88:139-46.
205. Harimaya K, Tanaka K, Matsumoto Y, Sato H, Matsuda S, Iwamoto Y. Antioxidants inhibit TNFalpha-induced motility and invasion of human osteosarcoma cells: possible involvement of NFkappaB activation. Clinical & experimental metastasis. 2000;18:121-9.
206. Mori T, Sato Y, Miyamoto K, Kobayashi T, Shimizu T, Kanagawa H, et al. TNFalpha promotes osteosarcoma progression by maintaining tumor cells in an undifferentiated state. Oncogene. 2014;33:4236-41.
207. van Etten B, de Vries MR, van IMG, Lans TE, Guetens G, Ambagtsheer G, et al. Degree of tumour vascularity correlates with drug accumulation and tumour response upon TNF-alpha-based isolated hepatic perfusion. British journal of cancer. 2003;88:314-9.
208. van der Veen AH, de Wilt JH, Eggermont AM, van Tiel ST, Seynhaeve AL, ten Hagen TL. TNF-alpha augments intratumoural concentrations of doxorubicin in TNF-alpha-based isolated limb perfusion in rat sarcoma models and enhances anti-tumour effects. British journal of cancer. 2000;82:973-80.
209. Yu Z, Geng J, Zhang M, Zhou Y, Fan Q, Chen J. Treatment of osteosarcoma with microwave thermal ablation to induce immunogenic cell death. Oncotarget. 2014;5:6526-39.
210. Gasparri A, Moro M, Curnis F, Sacchi A, Pagano S, Veglia F, et al. Tumor pretargeting with avidin improves the therapeutic index of biotinylated tumor necrosis factor alpha in mouse models. Cancer research. 1999;59:2917-23.
211. Palladino MA, Jr., Shalaby MR, Kramer SM, Ferraiolo BL, Baughman RA, Deleo AB, et al. Characterization of the antitumor activities of human tumor necrosis factor-alpha and the comparison with other cytokines: induction of tumor-specific immunity. J Immunol. 1987;138:4023-32.
212. Nawroth PP, Stern DM. Modulation of endothelial cell hemostatic properties by tumor necrosis factor. J Exp Med. 1986;163:740-5.
213. Hemmerle T, Probst P, Giovannoni L, Green AJ, Meyer T, Neri D. The antibody-based targeted delivery of TNF in combination with doxorubicin eradicates sarcomas in mice and confers protective immunity. British journal of cancer. 2013;109:1206-13.
214. Curnis F, Sacchi A, Borgna L, Magni F, Gasparri A, Corti A. Enhancement of tumor necrosis factor alpha antitumor immunotherapeutic properties by targeted delivery to aminopeptidase N (CD13). Nature biotechnology. 2000;18:1185-90.
215. Lu L, Li ZJ, Li LF, Wu WK, Shen J, Zhang L, et al. Vascular-targeted TNFalpha improves tumor blood vessel function and enhances antitumor immunity and chemotherapy in colorectal cancer. J Control Release. 2015;210:134-46.
216. Johansson A, Hamzah J, Payne CJ, Ganss R. Tumor-targeted TNFalpha stabilizes tumor vessels and enhances active immunotherapy. Proceedings of the National Academy of Sciences of the United States of America. 2012;109:7841-6.
217. Sacchi A, Gasparri A, Gallo-Stampino C, Toma S, Curnis F, Corti A. Synergistic antitumor activity of cisplatin, paclitaxel, and gemcitabine with tumor vasculature-targeted tumor necrosis factor-alpha. Clinical cancer research : an official journal of the American Association for Cancer Research. 2006;12:175-82.

218. Halama N, Michel S, Kloor M, Zoernig I, Benner A, Spille A, et al. Localization and density of immune cells in the invasive margin of human colorectal cancer liver metastases are prognostic for response to chemotherapy. Cancer research. 2011;71:5670-7.
219. Denkert C, Loibl S, Noske A, Roller M, Muller BM, Komor M, et al. Tumor-associated lymphocytes as an independent predictor of response to neoadjuvant chemotherapy in breast cancer. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2010;28:105-13.
220. DeNardo DG, Brennan DJ, Rexhepaj E, Ruffell B, Shiao SL, Madden SF, et al. Leukocyte complexity predicts breast cancer survival and functionally regulates response to chemotherapy. Cancer Discov. 2011;1:54-67.
221. Ray-Coquard I, Cropet C, Van Glabbeke M, Sebban C, Le Cesne A, Judson I, et al. Lymphopenia as a prognostic factor for overall survival in advanced carcinomas, sarcomas, and lymphomas. Cancer research. 2009;69:5383-91.
222. Tanaka H, Matsushima H, Mizumoto N, Takashima A. Classification of chemotherapeutic agents based on their differential in vitro effects on dendritic cells. Cancer research. 2009;69:6978-86.
223. Casares N, Pequignot MO, Tesniere A, Ghiringhelli F, Roux S, Chaput N, et al. Caspase-dependent immunogenicity of doxorubicin-induced tumor cell death. J Exp Med. 2005;202:1691-701.
224. Apetoh L, Ghiringhelli F, Tesniere A, Obeid M, Ortiz C, Criollo A, et al. Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. Nature medicine. 2007;13:1050-9.
225. Martins I, Kepp O, Schlemmer F, Adjemian S, Tailler M, Shen S, et al. Restoration of the immunogenicity of cisplatin-induced cancer cell death by endoplasmic reticulum stress. Oncogene. 2011;30:1147-58.
226. Menger L, Vacchelli E, Adjemian S, Martins I, Ma Y, Shen S, et al. Cardiac glycosides exert anticancer effects by inducing immunogenic cell death. Sci Transl Med. 2012;4:143ra99.
227. Obeid M, Tesniere A, Ghiringhelli F, Fimia GM, Apetoh L, Perfettini JL, et al. Calreticulin exposure dictates the immunogenicity of cancer cell death. Nature medicine. 2007;13:54-61.
228. Zappasodi R, Pupa SM, Ghedini GC, Bongarzone I, Magni M, Cabras AD, et al. Improved clinical outcome in indolent B-cell lymphoma patients vaccinated with autologous tumor cells experiencing immunogenic death. Cancer research. 2010;70:9062-72.
229. Curnis F, Sacchi A, Corti A. Improving chemotherapeutic drug penetration in tumors by vascular targeting and barrier alteration. J Clin Invest. 2002;110:475-82.
230. Brouckaert P, Takahashi N, van Tiel ST, Hostens J, Eggermont AM, Seynhaeve AL, et al. Tumor necrosis factor-alpha augmented tumor response in B16BL6 melanoma-bearing mice treated with stealth liposomal doxorubicin (Doxil) correlates with altered Doxil pharmacokinetics. International journal of cancer Journal international du cancer. 2004;109:442-8.
231. Manara M, Sinigaglia L. Bone and TNF in rheumatoid arthritis: clinical implications. RMD Open. 2015;1:e000065.
232. Osta B, Benedetti G, Miossec P. Classical and Paradoxical Effects of TNF-alpha on Bone Homeostasis. Front Immunol. 2014;5:48.
233. Sawicka M, Pawlikowski J, Wilson S, Ferdinando D, Wu H, Adams PD, et al. The specificity and patterns of staining in human cells and tissues of p16INK4a antibodies demonstrate variant antigen binding. PloS one. 2013;8:e53313.

234. Suzuki K, Bose P, Leong-Quong RY, Fujita DJ, Riabowol K. REAP: A two minute cell fractionation method. BMC Res Notes. 2010;3:294.
235. Park YB, Park MJ, Kimura K, Shimizu K, Lee SH, Yokota J. Alterations in the INK4a/ARF locus and their effects on the growth of human osteosarcoma cell lines. Cancer Genet Cytogenet. 2002;133:105-11.
236. Evangelou K, Bramis J, Peros I, Zacharatos P, Dasiou-Plakida D, Kalogeropoulos N, et al. Electron microscopy evidence that cytoplasmic localization of the p16(INK4A) "nuclear" cyclin-dependent kinase inhibitor (CKI) in tumor cells is specific and not an artifact. A study in non-small cell lung carcinomas. Biotech Histochem. 2004;79:5-10.
237. Romagosa C, Simonetti S, Lopez-Vicente L, Mazo A, Lleonart ME, Castellvi J, et al. p16(Ink4a) overexpression in cancer: a tumor suppressor gene associated with senescence and high-grade tumors. Oncogene. 2011;30:2087-97.
238. Nilsson K, Landberg G. Subcellular localization, modification and protein complex formation of the cdk-inhibitor p16 in Rb-functional and Rb-inactivated tumor cells. International journal of cancer Journal international du cancer. 2006;118:1120-5.
239. Shen WW, Wu J, Cai L, Liu BY, Gao Y, Chen GQ, et al. Expression of anion exchanger 1 sequestrates p16 in the cytoplasm in gastric and colonic adenocarcinoma. Neoplasia. 2007;9:812-9.
240. Chen YW, Chu HC, Ze-Shiang L, Shiah WJ, Chou CP, Klimstra DS, et al. p16 Stimulates CDC42-dependent migration of hepatocellular carcinoma cells. PloS one. 2013;8:e69389.
241. Goto T, Takano M, Hirata J, Kohno T, Ohtsuka S, Fujiwara K, et al. p16INK4a expression in cytology of ascites and response to chemotherapy in advanced ovarian cancer. International journal of cancer Journal international du cancer. 2009;125:339-44.
242. Robl B, Pauli C, Botter SM, Bode-Lesniewska B, Fuchs B. Prognostic value of tumor suppressors in osteosarcoma before and after neoadjuvant chemotherapy. BMC Cancer. 2015;15:379.
243. Kinoshita Y, Kalir T, Rahaman J, Dottino P, Kohtz DS. Alterations in nuclear pore architecture allow cancer cell entry into or exit from drug-resistant dormancy. Am J Pathol. 2012;180:375-89.
244. Widakowich C, de Castro G, Jr., de Azambuja E, Dinh P, Awada A. Review: side effects of approved molecular targeted therapies in solid cancers. Oncologist. 2007;12:1443-55.
245. Zitvogel L, Galluzzi L, Smyth MJ, Kroemer G. Mechanism of action of conventional and targeted anticancer therapies: reinstating immunosurveillance. Immunity. 2013;39:74-88.
246. Nakashima T, Kobayashi Y, Yamasaki S, Kawakami A, Eguchi K, Sasaki H, et al. Protein expression and functional difference of membrane-bound and soluble receptor activator of NF-kappaB ligand: modulation of the expression by osteotropic factors and cytokines. Biochemical and biophysical research communications. 2000;275:768-75.

10 Curriculum Vitae

Name ROBL Bernhard

Date of Birth 15.10.1987

Place of origin Vienna, Austria

E-mail Bernhard.robl@gmail.com

Education

Apr 2012 - Dec 2016	PhD Study in the Laboratory for Orthopedic Research, Balgrist University Hospital, Zurich, Switzerland within the "Cancer Biology" PhD Program of Life Science Zurich Graduate School, Faculty of Science, University of Zurich
Oct 2009 - Nov 2011	MSc in Molecular Bioengineering, Technische Universität Dresden, Germany Thesis Title: "Effects of flue gases on photosynthetic enzymes of microalgae"
Sep 2006 - Jun 2009	BSc in medical and pharmaceutical Biotechnology, IMC FH Krems - University of applied Sciences, Austria Thesis Title: "Development of angiogenesis assays"

Publications

Robl B, Botter SM, Boro A, Meier D, Neri D, Fuchs B. Evaluation of F8-TNF-α in a model of early stage and progressed murine osteosarcoma. Work in progress.

Robl B, Botter SM, Pellegrini G, Neklyudova O, Fuchs B. Evaluation of intraarterial and intravenous cisplatin chemotherapy in the treatment of metastatic osteosarcoma using an orthotopic xenograft mouse model. J Exp Clin Cancer Res. 2016 Jul 16;35(1):113.

Neklyudova O, Arlt MJE, Brennecke P, Thelen M, Gvozdenovic A, Kuzmanov A, **Robl B**, Botter S, Born W, Fuchs B. Altered CXCL12 expression reveals a dual role of CXCR4 in osteosarcoma primary tumor growth and metastasis. J Cancer Res Clin Oncol. 2016, 142(8):1739.

Boro A, Arlt MJ, Lengnick H, **Robl B**, Husmann M, Bertz J, Born W, Fuchs B. Prognostic value and in vitro biological relevance of Neuropilin 1 and Neuropilin 2 in osteosarcoma. Am J Transl Res. 2015, 7(3):640.

Robl B, Pauli C, Botter SM, Bode-Lesniewska B, Fuchs B. Prognostic value of tumor suppressors in osteosarcoma before and after neoadjuvant chemotherapy. BMC Cancer. 2015, 15:379.

Jentzsch T, **Robl B**, Husmann M, Bode-Lesniewska B, Fuchs B. Expression of MSH2 and MSH6 on a tissue microarray in patients with osteosarcoma. Anticancer Res. 2014, 34(12):6961.

Ram Kumar RM, Betz MM, **Robl B**, Born W, Fuchs B. ΔNp63α enhances the oncogenic phenotype of osteosarcoma cells by inducing the expression of GLI2. BMC Cancer. 2014, 14:559.

Jentzsch T, **Robl B**, Husmann M, Bode-Lesniewska B, Fuchs B. Worse prognosis of osteosarcoma patients expressing IGF-1 on a tissue microarray. Anticancer Res. 2014, 34(8):3881.

McHedlishvili L, Mazurov V, Grassme KS, Goehler K, **Robl B**, Tazaki A, Roensch K, Duemmler A, Tanaka EM. Reconstitution of the central and peripheral nervous system during salamander tail regeneration. Proc Natl Acad Sci U S A 2012, 109(34):E2258.

11 Acknowledgments

I would like to express my gratitude to Prof. Dr. Dr. **Bruno Fuchs** for providing me with the opportunity of conducting my doctoral work in his laboratory, for his support, advice, demonstrations and trust in my work.

I am thankful to my thesis director Prof. Dr. **Ian Frew** for kindly and optimistically guiding me through my doctoral studies as well as for being in my thesis committee, for his administrative support and for his scientific suggestions.

Furthermore, I would also like to show my respect and appreciation for my thesis committee members Prof. Dr. **Lubor Borsig** and Prof. Dr. **Dario Neri**, especially for their scientific expertise, their inspiring contributions as well as their bureaucratic support and supplies.

I am grateful to my supervisor Dr. **Sander Botter** for his supervision, availability and constant help throughout my PhD. Most of all, I would like to thank him for his patience during frequent discussions.

Special thanks go to Dr. **Ana Gvozdenovic**, Dr. **Aleksandar Boro**, Dr. **Matthias Arlt** and Dr. **Adam Sabile** for their generous help and advices throughout my PhD. They taught me a lot about solving biological problems and provided me technical help.

Many thanks also to all my current lab colleagues for their help, cheerfulness and the friendly atmosphere in the lab. Thank you, **Daniela, Olga, Joaquin and Alekhya,** for your help, for being friends and for cheering me up when times were tough or celebrating with me when times were good.

I would also like to thank my external collaborators, especially Dr. **Giovanni Pellegrini** for his profound knowledge in veterinary pathology, patience and for letting

me be his first customer. Also Dr. **Chantal Pauli** always had time for me including the weekends when I needed to discuss important issues about human sarcoma biology and **Philipp Probst**, who always provided me with endless supplies of antibodies and technical advice to successfully complete my work.

Without, external evaluation of doctoral issues, this work would not have been possible. Due to their objective opinions, friendly advices and great support during my spare time, I would like to thank **Laura, Tamara, Franziska, Sabine, Vinod, Johannes, Julian and Martin**.

My wholehearted gratitude belongs to my parents **Roswitha** and **Gerhard** for their love, great support across borders and for always supporting my work and ventures. Special thanks also to my only sister **Claudia**, her support, help in solving Austrian issues and the good times we have spent together.

Last but not least, I am expressing my deepest gratitude to my girlfriend and fiancé **Lisa** for exciting and fun times, for always being by my side and for sharing her life with me. I would like to specifically thank her for supporting me during stressful times, and motivating me in a way, that everything seemed possible.

Thank you so much.